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CORRIGENDA

In Gascoigne, J. A. & Gascoigne, M. M. (1960)
J. gen. Microbiol. **22**, 242–248.

On p. 243, Table 1: in *F. roseum*, source, 'Philadelphia Q.M. Depot, U.S.A. Q.M. 388'

for: 'Q.M. 388'

read: 'Q.M. 38g'

In Watkins, J. F. (1960). *J. gen. Microbiol.* **22**, 57–71.

On p. 57, 9 lines from bottom:

for: '...10% (v/v) lactalbumin hydrolysate in Hanks's...'

read: '...10% (v/v) lactalbumin hydrolysate and 20% (v/v) calf serum (Oxoid) in Hanks's...'

J. gen. Microbiol. (1960), **23**, 1–7
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Pigments of Trichophyta

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(*Received* 14 *October* 1959)

SUMMARY

Pigments possessing the properties of anthraquinones were isolated from three species of the genus *Trichophyton*. These pigments were characterized by their absorption spectra, their colour changes with various reagents, and their R_f values on paper chromatography. Most of these pigments were common to more than one species.

INTRODUCTION

It has been known for many years that various species of the genus *Trichophyton* can synthesize materials which diffuse into the culture medium to give characteristic colours (Tate, 1929; Thomson, 1943). Pinetti (1949) showed that the colouring matter of *T. violaceum* is a mixture of several closely related pigments; however, he achieved only a partial separation. Wirth, O'Brien, Schmidt & Sohler (1957), using column chromatography, isolated three pigments from *T. rubrum*, and found traces of a number of others. Mier (1957), by using paper chromatography and fractional precipitation, obtained similar results.

The pigments isolated were very similar to the substituted anthraquinones which have been isolated from species of the genus *Aspergillus*, *Penicillium*, and other sources (Tatum, 1944; Birkinshaw, 1953; Stickings & Raistrick, 1956). It was apparent that a more extensive survey of the trichophyton pigments was required as a preliminary to any work aimed at establishing their metabolic significance. It was also of more direct interest, since the pigmentation of colonies is used as a criterion of classification. The three species investigated and described here are *Trichophyton mentagrophytes*, *T. violaceum* and *T. rubrum*.

No attempt was made to elucidate the chemical structure of the pigments, and it was considered unnecessary to isolate large quantities of material: small agar cultures were used throughout. Fractional precipitation was used for the preliminary separation of the crude extracts, and paper chromatography provided a resolution of the pigments. Sufficient quantities of material were eluted from these chromatograms for the subsequent investigations.

METHODS

Organisms. Strains of each of the three species of fungi were obtained from two sources: the Department of Medical Mycology, Institute of Dermatology, London, and the Department of Medical Mycology, London School of Hygiene and Tropical Medicine. All cultures were grown on plates of Sabouraud's agar. The growth was harvested when a heavy pigmentation of the medium developed, but before any drying of the agar occurred.

Reagents were of A.R. grade. The light petroleum was of boiling range 60°–80°. Absorption spectra were plotted in chloroform solution ('Spectrosol', Hopkin and Williams, London) using a Hilger 'Uvispek' spectrophotometer.

Experimental procedure. The pigments were extracted from the agar into chloroform by the method of Mier (1957). Methods of fractionation of the individual extracts are discussed below. The volumes of light petroleum and methanol required for optimum separations varied slightly according to the relative quantities of the pigments present. Typical values for these volumes are given in the text. Paper chromatography was conducted by upward flow of solvent, the paper used being Whatman no. 3 MM. The chromatograms were viewed against an illuminated screen, or alternatively under ultraviolet light, the pigments then appearing as dark bands. When dealing with very small amounts of material, the paper was sprayed with a solution of magnesium acetate in ethanol, the pigments appearing as more intense, distinctively coloured bands (Shibata, Takito & Tanaka, 1950). The solvent systems used for chromatography were mixtures of light petroleum and chloroform. The effect of increasing the concentration of chloroform was to increase the R_f values of the pigments. Thus by adjusting the solvent system it was possible to achieve an optimum resolution of any particular group of pigments. R_f values were also found to be influenced slightly by the amount of material applied to the paper or by the presence of other compounds. Therefore, when establishing the identity of two or more pigments (as described below) they were first isolated by preliminary chromatograms. The smallest quantity of each pigment which could readily be observed was then placed on a fresh paper and re-run. The pigments were eluted from the paper with chloroform and the eluates were used for measuring the absorption spectra and for observation of the colour reactions. Blank sections of the chromatography paper were similarly eluted and their absorption spectra were measured.

Colour reactions of the pigments were carried out by pipetting small portions of the eluate into the depressions of a glazed porcelain tile, allowing to dry and then adding a few drops of either (*a*) glacial acetic acid, (*b*) N-sodium hydroxide, (*c*) N-sodium hydroxide saturated with sodium dithionite, or (*d*) concentrated sulphuric acid.

Criteria of purity and identity. Two criteria of purity were adopted: (*a*) when no further resolution of the pigments could be achieved by paper chromatography using a range of solvents; and (*b*) when no change in the absorption spectrum occurred following paper chromatography and elution, using at least two different solvents.

When pigments are reported identical, the following conditions were fulfilled: (*a*) the R_f values were identical over a range of solvents; (*b*) the absorption spectra were identical; (*c*) the colour reactions with various reagents were the same.

RESULTS

Trichophyton mentagrophytes. A separation of the pigments into two groups was achieved by the addition of 10 vol. of methanol to the concentrated chloroform extract, a red-brown flocculent precipitate being obtained. This was centrifuged, and the precipitate washed with a further quantity of methanol. The bright yellow supernatant was evaporated to a small volume and chromatographed. The pigment band remained homogeneous using a range of solvents. This pigment (A_1), was eluted and its absorption spectrum was plotted over the range 230–460 mμ. No change in the spectrum was observed on repeated chromatography using several solvents.

The precipitate from the methanol fractionation was re-dissolved in chloroform to give a bright orange-red solution. Chromatography again showed only one pigment (B) to be present. It was eluted and its absorption spectrum was plotted as before. A second fractionation from chloroform prior to chromatography caused no change in this curve.

Trichophyton violaceum. This contained a more complex mixture of pigments. The addition of 5 vol. of light petroleum to the concentrated chloroform extract effected a separation into two main groups, a brown precipitate being thrown down. The yellow supernatant was concentrated and chromatographed as for *T. mentagrophytes*; two pigments were found. The major component was a yellow compound (A_2), with a much smaller quantity of a yellow material running slightly ahead (A_1). These two pigments were eluted separately and purified by further chromatography to constant absorption spectra.

The brown precipitate from the first fractionation was washed with light petroleum and re-dissolved in chloroform. This solution was further fractionated by the addition of 10 vol. of methanol, and the red-brown precipitate thus obtained was washed in methanol, re-dissolved in chloroform and chromatographed. The final supernatant was concentrated and chromatographed, the optimum solvent system being 30% light petroleum and 70% chloroform. Five components could be distinguished on the chromatogram, but the quantities represented only a small fraction of the original extract. Two of these pigments (C_1 and C_2) were present in sufficient amount to be characterized by the methods described. In addition there were traces of an orange pigment running ahead of C_1, a yellow-orange pigment running between C_1 and C_2 and a red purple pigment between C_2 and the origin.

Trichophyton rubrum. A separation of the pigments from this species has been described previously (Mier 1957, Wirth *et al.* 1957). The R_f values of the pigments were measured for a range of solvents, and the colour reactions previously obtained were extended. Pure specimens of the pigments were used as markers when running chromatograms of material from other species.

Distribution of the pigments. One pigment (B) was found to be common to all three species. Pigment A_1 was common to *Trichophyton violaceum* and *T. mentagrophytes*, while pigments A_2, C_1 and C_2 of *T. violaceum* were found identical to the three pigments previously isolated from *T. rubrum* and described as A, C and 'yellow trace', respectively. *T. violaceum* showed traces of three other pigments not found in the other species. These results are summarized in Table 1. Essentially the same distribution of pigments was found in each strain of any one species; however, several strains of *T. mentagrophytes* ceased to synthesize anthraquinone pigments on repeated subculturing on Sabouraud's medium. In these subcultures a yellow pigment was produced which could not be extracted into chloroform and did not give any colour reactions with alkali. This pigment was not examined further. The results quoted for strains E. 5836 and E. 6302 were obtained using freshly isolated cultures. This lability of pigmentation was not shown by either *T. violaceum* or *T. rubrum*.

Table 1. *Distribution of pigments among the strains of fungi examined*

Fungus	Strain no.*	Pigment† A_1	A_2	B	C_1	C_2	Others
Trichophyton violaceum	7794	+	+ + +	+	+	+	Traces
	D 488	+	+ + +	+	+	+	Traces
	D 498	+	+ + +	+	+	+	Traces
T. rubrum	E 6606	.	+	+ +	+ +	+	.
	D 358	.	+ +	+ +	+	+	.
	D 360	.	+ +	+ +	+	+	.
T. mentagrophytes	E 5836	+ +	.	+ +	.	.	.
	E 6302	+ +	.	+ +	.	.	.

* The strains prefixed 'D' were obtained from the London School of Hygiene and Tropical Medicine, the remainder were isolated at the Institute of Dermatology.

† Pigment constitutes bulk of extract, + + + ; pigment present in appreciable quantity, + + ; pigment present in the minimum quantity for characterization, +.

Physical and chemical properties of the pigments. The R_f values of the pigments are shown in Table 2. The variation of R_f values with solvent composition is illustrated graphically in Fig. 1. The absorption spectra of the pigments are shown in Figs. 2–6. The source of the pigment is indicated on the diagrams. The eluate from sections of the chromatography paper was found to have a negligible absorption over the range investigated.

Table 2. *The R_f values of the pigments in various chloroform-light petroleum mixtures*

Percentage chloroform (v/v)	A_1	A_2	B	C_1	C_2
15	0·14	.	.	.	.
20	0·20	0·08	.	.	.
25	0·28	0·13	.	.	.
30	0·40	0·22	0·04	.	.
35	0·54	0·35	0·07	.	.
40	0·66	0·48	0·14	.	.
45	0·74	0·61	0·25	.	.
50	0·79	0·72	0·39	0·10	.
55	0·84	0·81	0·53	0·17	.
60	0·88	0·86	0·66	0·27	0·05
65	0·91	0·90	0·76	0·40	0·08
70	0·93	0·93	0·82	0·50	0·12
75	.	.	0·86	0·58	0·17
80	.	.	.	0·65	0·23
85	.	.	.	0·71	0·30
90	.	.	.	0·76	0·36

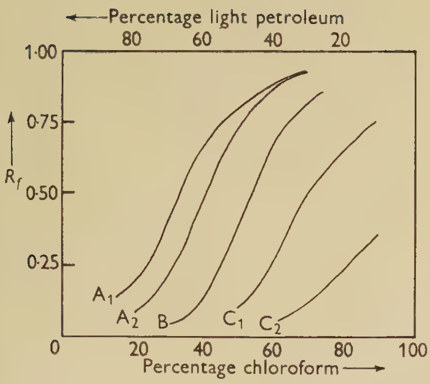


Fig. 1

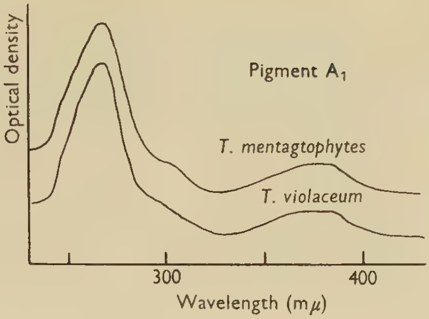
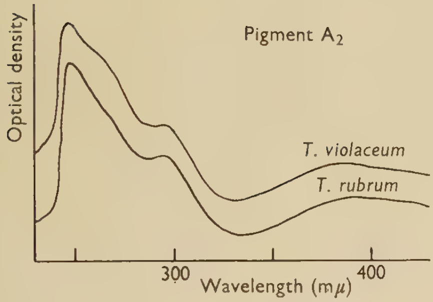


Fig. 2



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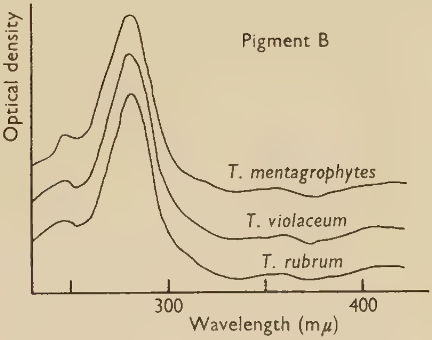

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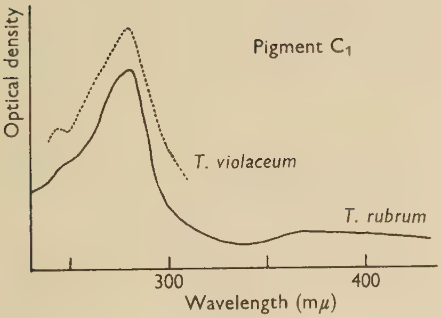

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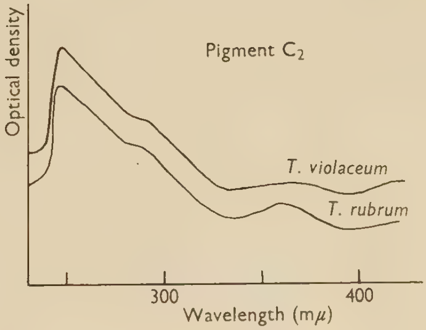

Fig. 6

Fig. 1. Variation of R_f values of the pigments with varying solvent composition.

Figs. 2–6. Ultraviolet absorption spectra of the pigments in chloroform solution.

Table 3. *Colour reactions of the pigments with various reagents*

Pigment ...	A_1	A_2	B	C_1	C_2
Solution in chloroform	Deep yellow	Bright yellow	Orange-red	Purple	Yellow
Solution in glacial acetic acid	Yellow	Yellow	Yellow	Wine red	Yellow
Solution in 1 N-sodium hydroxide	Mauve	Mauve	Purple	Blue	Yellow
Above solution saturated with sodium dithionite	Yellow	Yellow	Deep yellow	Amber	Purple
Solution in concentrated sulphuric acid	Peach red	Cherry red	Mauve	Green (slowly)	Orange
Colour on paper after magnesium acetate spray	Salmon pink	Raspberry	Orange	Blue	Mauve

DISCUSSION

The accumulation of pigments by the three species of *Trichophyton*, and the similarity of their distribution provide additional evidence for the botanical classification. Clearly the use of analytical techniques of this kind may be of use in diagnostic medical mycology.

Although no structures have been established for the compounds described, their chemical reactions and absorption spectra suggest that they are anthraquinone derivatives, many of which have been previously described. However, no evidence has been found of the isolation of these particular pigments from other sources.

The significance of the pigments is a matter of conjecture. Foster (1958) has pointed out that the accumulation of metabolites by the microfungi can be useful in elucidating new metabolic pathways. The oxidation–reduction properties of the trichophyton pigments suggest a role in hydrogen transport. It has been shown in this laboratory that under anaerobic conditions the pigments deposited in the medium are reduced by the fungi. In the presence of air re-oxidation takes place.

Raistrick and other workers have found that certain fungal anthraquinones possess powerful bacteriostatic properties. If bacteriostasis were their sole function, however, it is difficult to see why the property cannot be more generally demonstrated in this group of compounds. No definite answer can be given on the evidence at present available.

The authors wish to acknowledge the kindness of Miss B. M. Partridge, M.A., and Miss N. Dzawachiszwili, B.Sc., of the Institute of Dermatology, and Miss P. M. Stockdale, B.Sc., of the London School of Hygiene and Tropical Medicine, in providing the specimens of fungus.

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The Preparation and Properties of Spheroplasts of *Aerobacter aerogenes*

BY J. M. GEBICKI* AND A. M. JAMES
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(*Received* 13 *November* 1959)

SUMMARY

Cells of *Aerobacter aerogenes* growing in a nutrient medium were lysed by 1000 u. penicillin/ml. When the medium contained penicillin and sucrose, bacterial mass continued to increase on incubation, although no further cell division took place. After growth for 3 hr. in the presence of penicillin, all the rod-shaped bacteria were converted to fragile spheroplasts. The internal osmotic pressure of the spheroplasts was estimated by direct counts of the intact cells in solutions containing various concentrations of sucrose, sodium chloride or mixed phosphates. Measurements of optical densities of suspensions were unsuitable for the estimation of the numbers of spheroplasts. Morphological study suggested the existence of a spheroplast cell wall outside the protoplasmic membrane.

INTRODUCTION

Lysozyme digests the cell walls of several sensitive species of bacteria (Salton, 1953, 1954). Exposure of such organisms to lysozyme in hypertonic medium results in the release of the bacterial protoplast devoid of cell-wall material (Weibull, 1953; Gooder & Maxted, 1958; Dark & Strange, 1957; Brenner *et al.* 1958). These protoplasts are only stable in high osmotic pressure medium.

The main differences between bacteria and their protoplasts appear in the properties depending on the presence of a cell wall. The intact protoplasts are always spherical in shape and fragile to osmotic and mechanical shock (Weibull, 1953). Under very favourable conditions protoplasts have been observed to divide; the process is slower than with normal cells (McQuillen, 1955).

The preparation of spherical bodies of *Escherichia coli* exposed to high concentrations of penicillin in hypertonic media (Lederberg, 1956, 1957; Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957) gave experimental confirmation of the suggestion (Duguid, 1946) that penicillin interferes with the synthesis of bacterial cell walls while otherwise allowing growth to proceed. Like protoplasts, the penicillin-induced bodies are spherical and only stable in hypertonic media. Certain observations suggest, however, that penicillin does not completely remove the cell walls of sensitive bacteria (Tomcsik, 1954; Kandler, Hund & Zehender, 1958; Salton & Shafa, 1958). The process of reversible vacuolation exhibited by penicillin-

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induced 'spheroplasts' (Hurvitz, Reiner & Landau, 1958) of *Aerobacter aerogenes* suggests the existence of an envelope external to the protoplasmic membrane (Gebicki & James, 1958). Unlike protoplasts spheroplasts are capable of exponential growth and division (Lark, 1958). Spheroplasts share the fragility and osmotic properties of protoplasts but differ in their method of preparation (Salton, 1957; Chain, Duthie & Callow, 1945), growth and subsequent formation of normal viable bacteria (Lederberg, 1956).

Growth in the presence of penicillin alters the physical properties of the cell walls, but the nature of the alteration in terms of the chemistry of the structure is still obscure. The reason for the plasticity of the spheroplast envelope may lie in the inability of the cell to incorporate certain cell-wall materials in the presence of penicillin (Park & Strominger, 1957). On the other hand, a more important factor may be the alteration of the cross-linking rather than the amount of the cell-wall polymer.

In view of these uncertainties, it was considered useful to compare the properties of normal cells and spheroplasts of one organism. The surface properties of *Aerobacter aerogenes* have already been studied in this laboratory (James, 1957). The organism is not sensitive to lysozyme, even in the presence of versene (Gebicki & James, 1958; Repaske, 1958) so that comparative studies of its protoplasts were not possible. This paper gives an account of the preparation and properties of penicillin-induced spheroplasts of *Aerobacter aerogenes*. An account of their electrokinetic behaviour is published elsewhere (Gebicki & James, to be published).

METHODS

Organism and culture media. Cultures of *Aerobacter aerogenes* (NCTC, no. 240) were maintained by monthly subculture in Lab-Lemco broth (Oxoid). For the preparation of spheroplasts the medium was supplemented with magnesium sulphate (0·2 %) and sucrose (0·5 M). The cultures were incubated at 37° and usually aerated by gentle shaking. Cultures in defined media (Lowick & James, 1955) were incubated at 40° and aerated with a stream of sterile air.

Measurement of cell populations. Optical densities of cell suspensions were measured against the suspension medium in a Unicam S.P. 1400 spectrophotometer at 600 mμ. Cell counts were made in a haemocytometer (depth 0·02 mm.) with Thoma rulings.

Microscopy and photomicrography. Cell suspensions in wet films sealed between slide and coverslip were examined with a Reichert Neozet binocular microscope, equipped with a 'Polyphos' condenser. Phase contrast was usually employed. The same system was used for photomicrography.

Reagents. All reagents were of A.R. grade. Crystalline sodium salt of penicillin G (benzylpenicillin) of potency 1670 i.u./mg. (Glaxo Laboratories, Greenford, Middlesex) was stored in the cold before use.

Growth in hypertonic media. Synthetic medium. Rapidly growing cells were suspended in synthetic growth medium and in the same medium supplemented with either glucose (0·7 M), sodium chloride (1·0 M), magnesium sulphate (1·0 M) or mixed sodium and potassium phosphates (0·5 M). The suspensions were incubated without aeration and the optical densities measured at frequent intervals.

Nutrient medium. Cultures in nutrient broth supplemented with sucrose (0·7 M) or glucose (0·7 M) were incubated at 37° and aerated by shaking. Controls, in which the high concentrations of sugar were omitted, were also included.

The lytic action of penicillin. Cultures rapidly growing in nutrient medium, supplemented with 0·2 % magnesium sulphate, were incubated with various concentrations of penicillin (0–1000 u./ml.). Optical densities of samples removed at intervals were determined.

Growth in the presence of penicillin and sucrose. A young culture containing $7 \cdot 5 \times 10^8$ cells/ml. was diluted fivefold into fresh nutrient medium containing sucrose and penicillin (0·6 M and 1000 u./ml. respectively). Samples were removed during subsequent incubation at 37° and their optical densities and cell counts determined. The cultures were aerated by mild shaking.

Standard method for the preparation of spheroplasts. A young (8 hr.) bacterial culture containing about 10^9 cells/ml. was diluted fivefold into fresh nutrient broth containing magnesium sulphate (0·2 %), sucrose (0·5–0·6 M) and penicillin (500–2000 u./ml.). After 3 hr. incubation with gentle shaking at 37° the culture was cooled and the spheroplasts harvested at 0° in the pre-cooled centrifuge. The collected spheroplasts were covered quickly with cold suspension medium and resuspended by gentle agitation. Shaking and warming were avoided. For overnight storage, the spheroplasts were suspended in buffered 1·0 M-sucrose or 0·5 M-sodium chloride and kept at 0°.

Thermal fragility of the spheroplasts and the effect of formalin. The spheroplasts were harvested and resuspended in 0·2 M solutions of sucrose in M/300 mixed phosphate buffer at pH 7·0. One half of the suspension was allowed to stand at room temperature and the other was warmed rapidly to 65°. Changes in the optical densities of the suspensions were followed for 10 min. Samples were examined under phase contrast before and after heating.

Two portions of a similar preparation of spheroplasts in 1·0 M-sucrose at pH 7·0 were diluted into 20 vol. of the buffered sucrose solution at 0°. Formalin was added to one portion (final concentration 0·5 %) and both were quickly heated to 60°. Numbers of the intact cells in each sample were determined at intervals. In addition the samples were examined under phase contrast.

Photomicrography of the spheroplasts. Drop samples of bacterial cultures incubated with penicillin in a hypertonic medium were placed between a slide and coverslip and examined under phase contrast. Typical stationary organisms were photographed. No dyes or killing agents were added to the suspensions.

The internal osmotic pressure of the spheroplasts. (*a*) *Optical densities of suspensions.* Spheroplasts were suspended in solutions containing various concentrations of sodium chloride or sucrose buffered at pH 7·0. Changes in the optical densities of suspensions were followed, frequently over several hours.

(*b*) *Lysis in sucrose solutions.* 0·5 ml. portions of a thick suspension of spheroplasts in 1·0 M-sucrose were diluted into 9·5 ml. of solutions containing various concentrations of sucrose at pH 7·0. The suspensions were incubated at 25 ± 0·1°, samples withdrawn at intervals, treated with formalin, to preserve the spheroplasts, and cell counts determined.

(*c*) *Lysis in sodium chloride solutions.* Spheroplasts in 0·5 M buffered solution of sodium chloride were diluted with salt solutions at pH 7·0. The final concentrations of sodium chloride were 0·05–0·5 M. The numbers of intact spheroplasts remaining in suspension after incubation at 25° were determined by direct counting.

(*d*) *Lysis in mixed phosphate buffer solutions.* In a similar experiment, the spheroplasts were incubated in solutions containing various concentrations of phosphates. These were obtained by mixing and diluting equal volumes of M/3 disodium hydrogen phosphate and potassium dihydrogen phosphate. Intact spheroplasts were counted as previously.

Optical properties of suspensions of spheroplasts. Spheroplasts were suspended in hypertonic solutions and changes in the optical densities of the suspensions with time were measured. The variables investigated were the osmotic pressure, pH and electrolyte contents of the solutions.

RESULTS

Penicillin lyses cells of *Aerobacter aerogenes* growing in a simple synthetic medium (Gebicki, 1959), but the high solute concentrations required to preserve any formed spheroplasts prevented cell division altogether. Since the antibiotic acts only on actively growing bacteria, the synthetic medium could not be used for the preparation of spheroplasts.

On the other hand, the mean generation time of the nutrient broth cultures containing 0·7 M-sucrose were the same as those of control suspensions (30 min.). This was even reduced when glucose was the added solute, but as sucrose is the most commonly used substance for the stabilization of protoplasts, it was used in further experiments.

The lytic action of penicillin on cells growing in the nutrient medium is shown in Fig. 1. The rate of growth is affected by penicillin concentrations above 10 u./ml. At 100 u./ml., normal growth was restored after 4 hr. incubation. The initial increase of the optical densities of suspensions containing the highest concentrations of penicillin used indicates that the mass of cells is approximately doubled before the onset of lysis.

The nutrient medium was suitable for the production of spheroplasts since it did not prevent bacterial growth when supplemented with high concentrations of sucrose and, further, suitable amounts of penicillin lysed the cells growing in it. In Fig. 2 the mass of cells growing in the medium containing sucrose in the presence of 1000 u./ml. of penicillin is compared with the actual number of cells per ml. of suspension. A series of photomicrographs illustrate a typical sequence of changes which the normal cells (Pl. 1, fig. 1) undergo under the influence of penicillin. Characteristic protoplasmic protrusions were formed 30 min. after the exposure to penicillin (Pl. 1, figs. 2, 3). Further incubation led to the increase of the size of the protrusions (Pl. 1, figs. 4, 5), detachment of the rod-shaped fragments and the appearance of characteristic vacuolated spheroplasts (Pl. 2, figs. 6–8). When the external osmotic pressure was carefully lowered by the addition of water, the protoplasm expanded in the initially vacuolated spheroplast and, after breaking through the confining barrier, formed a spherical protrusion outside the spheroplast proper (Pl. 2, fig. 9). The protrusion then expanded at the expense of the spheroplast, the protoplasm gradually leaving the original cell. After a short time (2–3 min.) the new sphere burst leaving no clearly defined structure. A normal 'ghost' was left by the spheroplast (Pl. 2, fig. 10). Frequently the whole process was so rapid that the change from spheroplast to 'ghost' appeared to proceed directly. However, the controlled lysis was observed on numerous occasions.

The process of vacuolation could be reversed by placing the spheroplasts in media of lower osmotic pressure. On subsequent addition of sucrose or sodium chloride, the vacuoles formed again. This could be repeated several times.

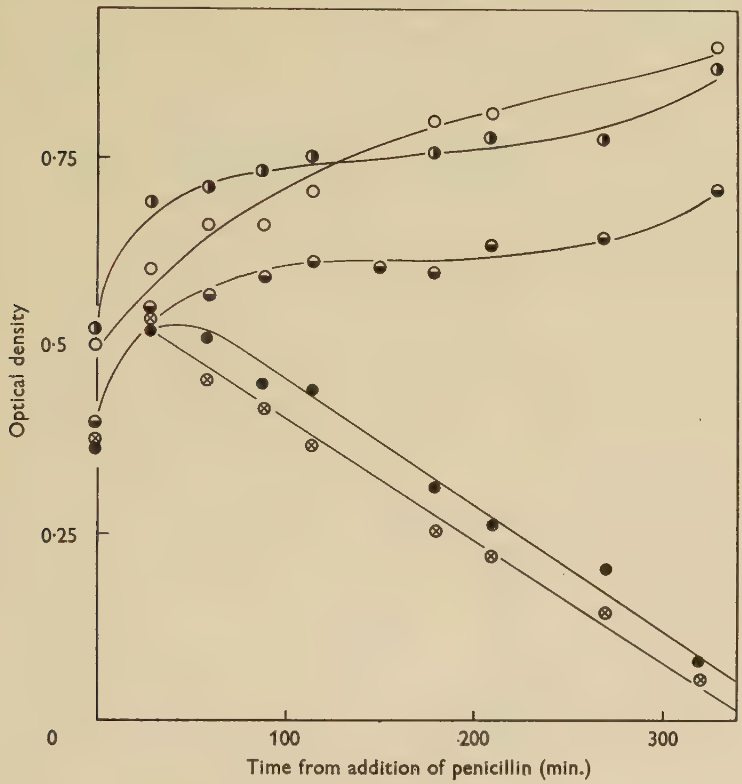

Fig. 1. The lytic effect of penicillin in nutrient media. ○, Control; ◑, 10 u. penicillin/ml.; ◒, 100 u. penicillin/ml.; ●, 1000 u. penicillin/ml.; ⊗, 2000 u. penicillin/ml.

The lack of correlation between the actual numbers of intact spheroplasts and the optical density of the suspension is shown in Fig. 3. The optical density curve suggests a continuous decrease in the number of cells which does not actually occur. Several other experiments, in which the concentration of the stabilizing solute was the independent variable, emphasized the unsuitability of the method for the determination of the spheroplast populations.

The effectiveness of sucrose and sodium chloride in the preservation of intact spheroplasts at different osmotic pressures is shown in Fig. 4. The figure taken as 100 % preservation for the given solute was obtained by averaging the numbers of spheroplasts per unit volume of suspension after 1 min. standing in solution of osmotic pressure above 5 atmospheres. Values above 90 % must be regarded as complete preservation in view of the errors involved. Comparative studies for sodium and potassium phosphates were not made because of the difficulty of obtaining accurate values for the osmotic pressures of their solutions. Some indication of the stabilizing action of the phosphates is seen in Fig. 5. Spheroplasts suspended in M/6 buffer solution were counted before and after storage for 24 hr. at 0° C.; the counts were 279 and 234 × 10^6 cells/ml. of suspension respectively.

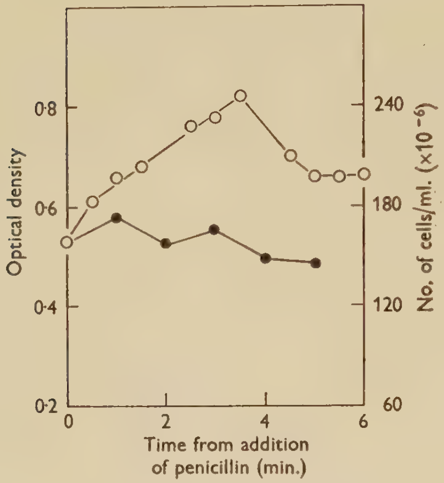

Fig. 2

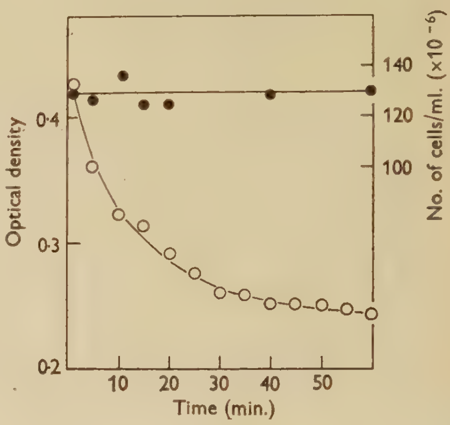

Fig. 3

Fig. 2. Growth of *Aerobacter aerogenes* in presence of sucrose and penicillin. ○, Optical density, i.e. bacterial mass; ●, no. of cells.

Fig. 3. Spheroplasts suspended in 7 % sucrose at pH 6·8 and 20° C. ○, Optical density; ●, no. of cells.

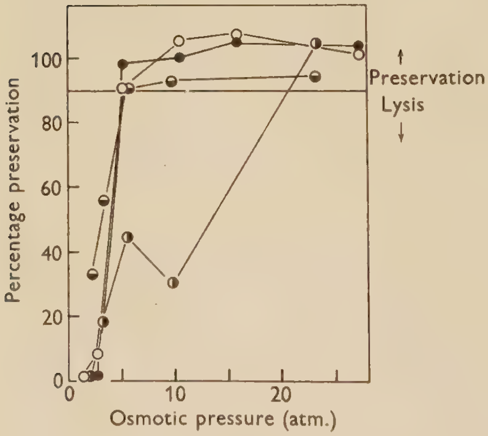

Fig. 4

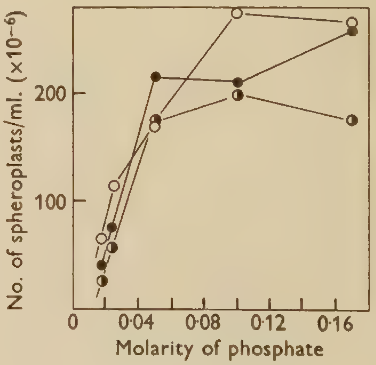

Fig. 5

Fig. 4. Stability of spheroplasts in solutions of sucrose and sodium chloride at 25° C. Sucrose, ○, after 60 min., ●, after 20 hr.; NaCl, ⊖, after 60 min., ◑, after 23 hr.

Fig. 5. Stability of spheroplasts in mixed phosphate buffer solutions at 25° C. ○, After 5 min., ●, after 30 min., ◑, after 18 hr.

An attempt to discover the factors responsible for the anomalous optical properties of suspensions of spheroplasts is summarized in Table 1. Changes in the optical densities of the suspensions were followed under identical conditions for 1 hr. All solutions were buffered with mixed phosphates unless otherwise stated. Since it has been demonstrated (Fig. 3) that a decrease in the optical density of 46 % can occur without lysis, values less than this figure can be assumed to be due to factors other than destruction of the spheroplasts. This was, in fact, confirmed by microscopic examination of cell suspensions. Some cell damage occurred at pH 7·5, both at 20° and at 37°. Trypsin had no accelerating effect on lysis. A certain regularity of the pattern of changes in the optical densities of suspensions with pH suggests that even at pH 7·5 lysis may be fairly rapid. An experiment in which a wide range of pH values was used suggested that at neutral pH there was no lysis, while below pH 4·3 the optical densities of suspensions increased. Spheroplasts in acid buffers appeared as highly refracting irregular bodies and they did not lyse on resuspension in distilled water. Spheroplasts suspended at pH 3·5 for 1 hr., collected by centrifugation and resuspended at pH 6·7, behaved normally, indicating that the effect of the acid was reversible.

Table 1. *Relationship between lysis and percentage lowering of the optical densities of suspensions of spheroplasts*

Suspension medium	Temp. (° C.)	pH	% lowering of optical density after 1 hr.	Lysis
7 % sucrose	20	6·8	46	–
10 % sucrose	37	7·5	57	(+)
10 % sucrose + trypsin	37	7·5	54	(+)
10 % sucrose	20	7·5	50	(+)
10 % sucrose	20	3·4	–47	–
10 % sucrose + pepsin	20	3·4	–14	–
20 % sucrose + phosphate buffer	20	6·7	32	–
20 % sucrose + citrate buffer	20	6·7	27	–
7 % sucrose + phosphate buffer	20	6·8	41	–
7 % sucrose + citrate buffer	20	6·8	32	–

(+), Slight lysis confirmed by microscopic examination.

DISCUSSION

The term spheroplasts has been suggested to describe abnormal bacterial forms fragile to osmotic and mechanical shock, spherical in shape and released by the action of penicillin on the rapidly growing cells: spheroplasts of *Escherichia coli* lyse rapidly when warmed to 56° (Hurwitz *et al.* 1958). The description of the fragile, spherical forms of *Aerobacter aerogenes* as spheroplasts can be justified on several grounds.

Normal growth precedes lysis of cells exposed to penicillin during a period corresponding to the doubling of the bacterial mass in suspension (Fig. 1). That this is not accompanied by actual cell division is shown by direct cell counts (Fig. 2). Thus, during the initial period of exposure to the inducing agent, the cell-growth mechanism is not damaged, but division ceases. Growth continues, at a diminished rate, for a further 3 hr. During this time the individual cells undergo a series of changes which result in the production of the spherical forms. The synthesis of cell material is counterbalanced by a process of gradual lysis, the optical density of the culture becoming constant about 5 hr. after the initial exposure to penicillin.

The internal osmotic pressure of the spheroplasts lies between 5 and 6 atmospheres at 25° C. This compares well with the value for *Escherichia coli* estimated by a different method (Mitchell & Moyle, 1956), and suggests that the spheroplast envelopes have decreased tensile strength. The superiority of sucrose in the stabilization of spheroplasts can be explained if it is assumed that the cells possess a mechanism for the active transport of K^+ and PO_4^{---}.

Various workers have confirmed a direct relation between lysis and turbidities of suspensions of protoplasts and bacteria (Mitchell & Moyle, 1956; Salton, 1957; Christian & Ingram, 1959). Turbidity measurements, however, cannot be applied to the estimation of the numbers of spheroplasts remaining in suspension. The most highly organized fragments visible after lysis of bacteria by lysozyme are the cytoplasmic membranes and the associated lipid granules. These 'ghosts' are difficult to observe under the microscope (Weibull, 1955). In contrast, the spheroplast 'ghosts' appear to be more dense optically (Pl. 2, fig. 10) and they frequently flocculate. Extensive vacuolation of the spheroplasts in response to the external osmotic conditions may also explain their unexpected optical properties. Bacteria are difficult to plasmolyse and the extent of shrinkage is usually slight (Weibull, 1956). Volumes of protoplasts vary slightly with the external conditions, the osmotically inert fraction of the total volume being 36% for protoplasts of *Bacillus megaterium* (Weibull, 1955) and 76% for those of *Micrococcus lysodeikticus* (Gilby & Few, 1959). The process of vacuolation of the spheroplasts is rather slow; this probably accounts for the continuous changes in the optical densities of their suspensions (Fig. 3).

Lysis of spheroplasts at high temperatures and at values of pH greater than 7·0 must be the result of a rupture in the protoplasmic membrane. In alkaline conditions the membrane constituents are probably slowly denatured, the process resulting in alteration and breakdown of the structure. Such changes do not occur in acid conditions, but it is surprising to note the profound alteration of the physical properties of the protoplasm at pH values below 4·3. Resistance of the acid-treated protoplasts to lysis is probably due to the coagulation of the cell contents. The process is reversible even after prolonged standing at pH 3·5. It is interesting to speculate whether such changes occur in normal bacteria, whose viability may not be affected by acid treatment.

The swellings and protrusions observed in bacteria exposed to penicillin in hypertonic media are first visible after 30 min. incubation, i.e. at the time corresponding to the onset of osmotic fragility. The protrusions appear in the middle of the bacteria, where the cells would be expected to divide. Protrusions continue to grow, until finally the remaining rod-shaped fragments become detached, leaving the large spheres. In 5 hr. the surface area of a normal cell converted to a spheroplast increases by a factor of about 70.

The presence of an envelope around the spheroplasts of *Aerobacter aerogenes* which can be differentiated from the protoplasmic membrane is suggested by the examination of the vacuolated bodies. The protoplasm must at all times be surrounded by a hydrophobic membrane if lysis is not to take place. In hypertonic solution, the membrane becomes detached from the inside of the envelope which surrounds the spheroplast and which remains clearly visible (Pl. 2, fig. 8). The vacuole then occupies the space between the protoplasmic membrane and the spheroplast cell wall. The two structures can be demonstrated by observing the emergence of a protoplasmic extrusion from within the spheroplast (Pl. 2, fig. 9). Bacterial protoplasts are surrounded by the protoplasmic membranes only and do not develop vacuoles. Although their size varies slightly with the external osmotic pressure, they remain spherical in shape. It is interesting to note that many authors have observed the presence of vacuoles in spheroplasts, without commenting on the implication of the phenomenon (Lederberg, 1956; Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957; Lark, 1958, McQuillen, 1958).

The results obtained support the hypothesis that bacterial spheroplasts are surrounded by a modified form of cell wall in addition to the protoplasmic membrane. This is further confirmed by a comparison of the electrokinetic properties of the normal cells and spheroplasts (Gebicki & James, to be published).

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EXPLANATION OF PLATES

Anoptral Phase Contrast, 3500

PLATE 1

Fig. 1. Normal cells of *Aerobacter aerogenes*.

Figs. 2, 3. After 30 min. incubation with sucrose and penicillin.

Fig. 4. After 60 min.

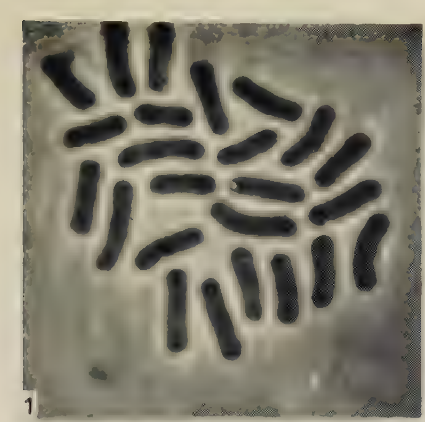
Fig. 5. After 2 hr.

PLATE 2

Figs. 6–8. Vacuolated spheroplasts.

Fig. 9. Protoplast emerging from within a spheroplast, only after careful water treatment.

Fig. 10. Spheroplast ghost, after extreme dilution with water.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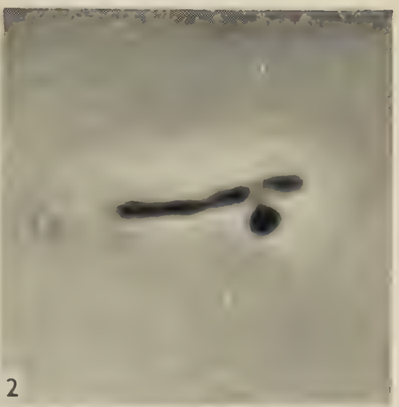
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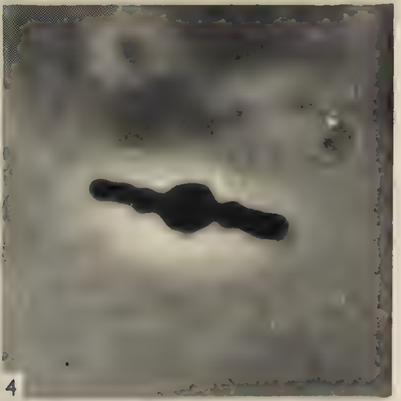
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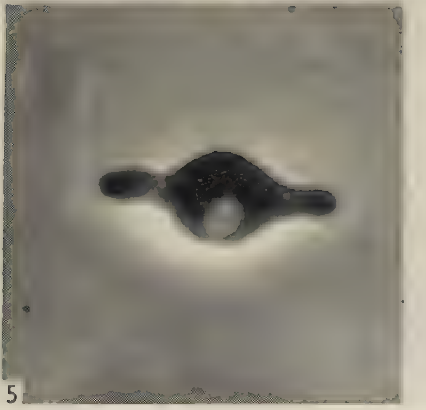
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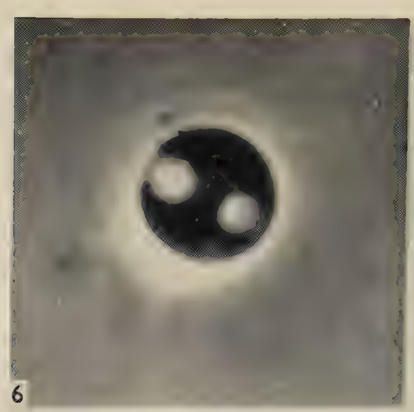
J. M. GEBICKI AND A. M. JAMES (*Facing p.* 18)



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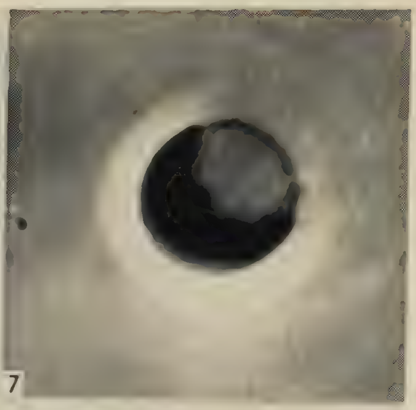

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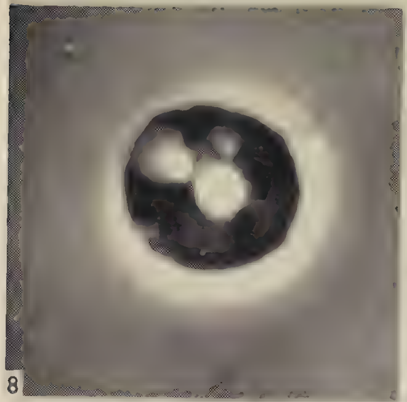

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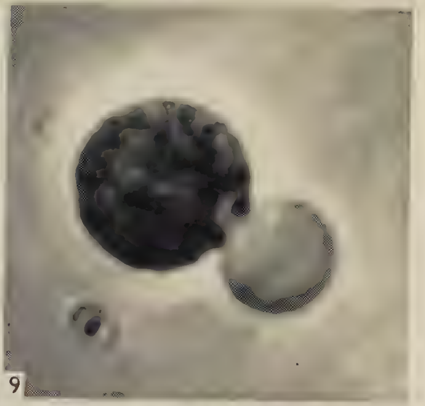

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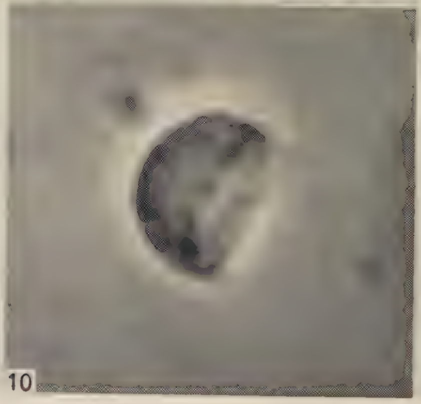

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J. M. GEBICKI AND A. M. JAMES

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Printed in Great Britain

Lysis of Protoplasts of *Micrococcus lysodeikticus* by Ionic Detergents

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(*Received 7 December* 1959)

SUMMARY

The time course of lysis of protoplasts of *Micrococcus lysodeikticus* was studied by spectrophotometric methods. The lytic agents used were sodium dodecylsulphate and a range of cationic detergents which each possessed a n-C_{12} alkyl chain and comprised the amine hydrochloride, the trimethylammonium bromide, the pyridinium bromide and the quinolinium bromide. Also the inhibition of lysis by uranyl ions and the action of detergents on isolated protoplast membranes were investigated. It is concluded that the cationic detergents act on the complex phosphatidic acid lipid component of the protoplast membrane and that lysis results from secondary osmotic effects. Access to the reactive site by agents of similar hydrophilic-hydrophobic balance is controlled by steric factors. Anionic detergents, however, cause direct disintegration of the protoplast, membrane independent of osmotic stresses, probably by interaction with the membrane protein moiety; a concurrent interaction with lipid, possibly through a lipoprotein complex, is not definitely excluded.

INTRODUCTION

There is now a considerable body of evidence to implicate the bacterial cytoplasmic membrane as the seat of the bactericidal action of ionic detergents. This evidence was reviewed by Salton (1957) and by Pethica (1958). The differences in response as between Gram-positive and Gram-negative bacteria have been ascribed to differences in lipid content in the cell walls of the bacteria. After the initial disruption of the permeability properties of the cell membranes which is always associated with the bactericidal action, full lysis and dispersion of the structural components of the cells may follow through processes not necessarily directly connected with the detergent. In some circumstances the continuing autolytic disintegration can be inhibited by high detergent concentrations (Dawson, Lominski & Stern, 1953). With the development of the now well-established technique of protoplast preparation by the action of lysozyme (Tomcsik & Guex-Holzer, 1952; Weibull, 1953) it became possible to study the action of detergents directly on the protoplast membrane of some bacteria in the absence of cell wall. In earlier work (Gilby & Few, 1957*a*, *b*) qualitative and quantitative parallels were established between the bactericidal action of detergents and their lysis of protoplasts. With the dodecyl series of detergents, the lytic action with protoplasts of *Micrococcus lysodeikticus* was in the sequence

$$C_{12}H_{25}-NH_3^+ > -N(CH_3)_3^+ > -SO_4^- > -SO_3^-.$$

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It was concluded that in both the bactericidal action and the lysis of protoplasts the site of action of ionic detergents was a lipid component of the protoplast membrane. Chemical analysis (Gilby, Few & McQuillen, 1958) showed that the protoplast membrane of *M. lysodeikticus* contained 28 % lipid which consisted mainly of a complex phosphatidic acid with properties consistent with the above interpretation. Salton (1957) investigated the action of detergents on protoplasts of *Bacillus megaterium*. In the present paper further investigations into the action of ionic detergents on protoplasts of *M. lysodeikticus* and on isolated protoplast membranes will be described. A wider range of cationic detergents than previously was used to determine the importance of steric factors, and the effects of pretreatment with uranyl ions on the lysis of protoplasts by anionic and by cationic detergents were studied.

METHODS

Bacteria. Micrococcus lysodeikticus (NCTC 2665, the strain used previously) was grown on a beef tryptic-digest agar medium for 20 hr. at 37°. The organisms were harvested and washed with distilled water as previously described.

Preparation of protoplasts and protoplast membranes. The procedures used were those described in earlier work (Gilby & Few, 1957*a*, *b*; Gilby *et al.* 1958). Protoplasts were prepared by the action on the organisms (at equiv. 10 mg. dry wt./ml.) of lysozyme (100 μg./ml.) in a medium containing M-sucrose and 0·05 M-NaCl. The resulting protoplasts were centrifuged down and, after the supernatant fluid was decanted, were gently resuspended in the sucrose + sodium chloride medium. Protoplast membranes were prepared from lysozyme-induced lysates in 0·1 M-NaCl by centrifugation at 20,000 *g* and washed in 0·05 M-NaCl.

Ionic detergents. Sodium dodecylsulphate (SDS) and dodecylamine hydrochloride (DAC) were pure samples kindly supplied by Dr E. Hutchinson and Professor E. Stenhagen, respectively. Dodecyltrimethylammonium bromide (DTAB), dodecylpyridinium bromide (DPB) and dodecylquinolinium bromide (DQB) were prepared from the reaction of dodecyl bromide with the corresponding tertiary base. Pure DTAB and DPB were obtained by simply mixing the reactants followed by crystallization; the conditions for preparing and purifying DQB, a new compound, were given in Few, Gilby, Ottewill & Parreira (1958).

Spectrophotometric determination of lysis. The optical density at wavelength 500 mμ was measured at known time intervals during reaction with the detergents. The concentration of protoplasts corresponded to 0·5 mg. bacterial dry wt./ml., and the reaction medium (pH 6·3 ± 0·1) contained M-sucrose + 0·05 M-NaCl. The reaction temperature was 22 ± 1°. In the range of protoplast and detergent concentrations used, the rate of lysis was independent of protoplast concentration. With protoplast membranes, the suspension medium was the same as for protoplasts and the membrane concentration was adjusted to give an initial optical density of 0·25.

Pretreatment of protoplasts with uranyl nitrate. Protoplasts to give equiv. 6 mg. dry wt./ml. were suspended in samples of the usual sucrose + NaCl medium which also contained uranyl nitrate at different concentrations. After 30 min. at 20–22°, the protoplasts were centrifuged down, resuspended in the standard sucrose + NaCl medium and re-centrifuged. Finally they were resuspended in the standard medium, this stock suspension being adjusted to an optical density corresponding to 3·0 mg. wt./ml. Lysis with detergents was then carried out as above.

RESULTS

Protoplast lysis by cationic detergents

The results of lysis with DAC and DTAB have already been published (Gilby & Few, 1957*b*). The optical density/time curves for lysis with DPB and DQB are shown in Figs. 1 and 2. The general form of the lysis curves is similar with each of the four detergents; at the highest concentrations used precipitation of intracellular constituents occurred with each. This was important only for DAC since, for the other agents, lysis was complete at concentrations lower than those at which precipitation began. To compare the results, as previously (Gilby & Few, 1957*a*), a standard time of lysis of 30 min. was adopted and the decrease in optical density plotted against detergent concentration (Fig. 3). From each of these curves can be estimated the protoplast lytic concentration, again defined as that concentration of detergent which, in 30 min., decreased the optical density of a protoplast suspension by 90 % of the loss observed on complete lysis; the values are given in Table 1. The protoplast lytic concentration for DAC was obtained by extrapolation of the steep curve.

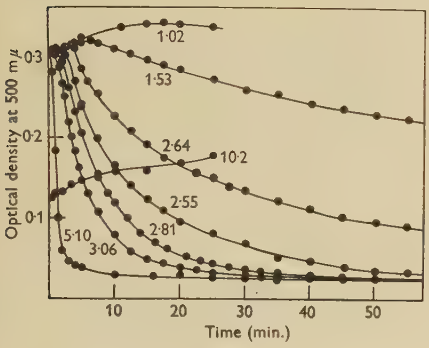

Fig. 1

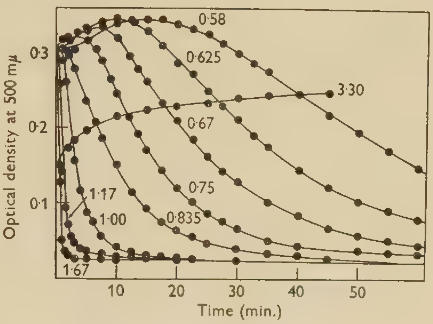

Fig. 2

Fig. 1. Lysis of *Micrococcus lysodeikticus* protoplasts by dodecylpyridinium bromide in M-sucrose + 0·05 M-NaCl solution at concentrations of DPB ($M \times 10^4$) given on curves.

Fig. 2. Lysis of protoplasts by dodecylquinolinium bromide in M-sucrose + 0·05 M-NaCl at concentrations of DQB ($M + 10^4$) given on curves.

Table 1. Micrococcus lysodeikticus, *protoplast lytic concentrations of cationic detergents*

Detergent	Lytic concen. for protoplasts ($C \times 10^4$ M)	Activity relative to DAC, i.e. $C_{DAC}/C_{\text{detergent}}$
DQB	0·77	2·34
DAC	1·8	1·00
DPB	2·6	0·69
DTAB	3·2	0·56

Action of detergents on protoplast membranes

The action of DTAB and of SDS on suspensions of protoplast membranes in 0·05 M-NaCl was followed by measuring changes with time of the optical density at 500 mμ. With DTAB, even at concentrations up to 10 times the protoplast lytic concentration, there was no change up to 12 hr. With SDS, however, the lysis curves were similar to those already published (Gilby & Few, 1957*b*) for reaction of SDS with protoplasts, both in the range of SDS concentration over which reaction occurred and in the shape of the curves. Thus there was an initial rapid decrease in optical density followed by a period of at least 12 hr. during which further decrease in optical density was very slow. The extent of the initial rapid decrease in optical density depended on the SDS concentration.

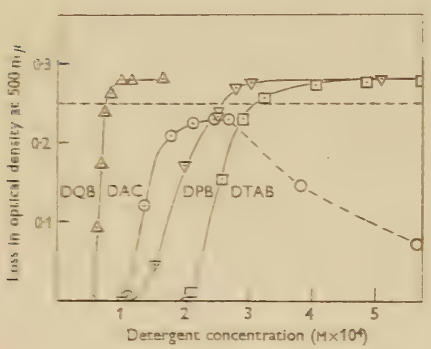

Fig. 3

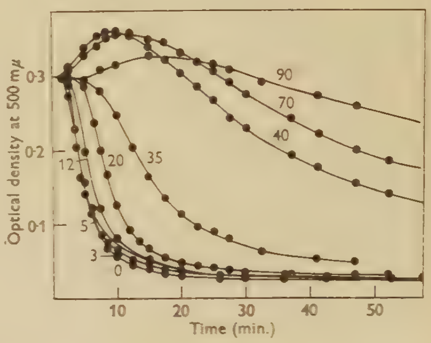

Fig. 4

Fig. 3. Decrease in optical density of protoplast suspensions after 30 min. lysis; plotted as function of cationic detergent concentration. Broken line parallel to abscissa indicates 90 % lysis.

Fig. 4. Lysis of protoplasts pretreated with uranyl nitrate by dodecyltrimethylammonium bromide in M-sucrose + 0·05 M-NaCl solution. Concentration of DTAB = 4·0 × 10^{-4}M. Concentrations of uranyl nitrate (M × 10^5) given on curves.

Inhibition of protoplast lysis by pretreatment with uranyl nitrate

Pretreatment of protoplasts with uranyl nitrate affected the course of lysis in a manner similar to a decrease in detergent concentration. In Fig. 4 the optical density time curves are given for lysis by DTAB of protoplasts pretreated with uranyl nitrate at different concentrations. These curves are similar in shape to those obtained with cationic detergents and with protoplasts without uranyl ion pretreatment but where different detergent concentrations are used. The highest uranyl nitrate concentrations even induced the initial increase in optical density observed at low cationic detergent concentrations. Lysis of uranyl nitrate pretreated protoplasts with SDS gave the results shown in Fig. 5. With SDS also, the uranyl nitrate pretreatment had the same effect on the lysis time curves as a decrease in detergent concentrations (Gilby & Few 1957*b*). To demonstrate the influence on lysis of the concentration of uranyl nitrate with which protoplasts had been pretreated, the optical density at 500 mμ after a standard time of 30 min. lysis was plotted (Fig. 6) as a function of uranyl nitrate concentration. With DTAB and with SDS there was a critical range of concentration of uranyl nitrate (30–40

$\times 10^{-5}$ M) over which there occurred a very sharp increase in the inhibition of lysis. This same critical concentration range can be shown by the application of other criteria to the results. Observations of the threshold concentration of uranyl nitrate which caused agglutination of protoplast suspensions showed that this was also in the range $30–40 \times 10^{-5}$ M. This critical range of uranyl nitrate concentration, important in both the lysis and coagulation of protoplasts, is considerably less than the concentration of *c.* 10^{-2} M needed to reverse the charge as found by electrophoresis (Few, Gilby & Seaman, 1960).

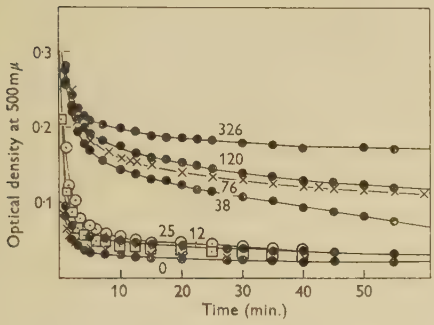

Fig. 5

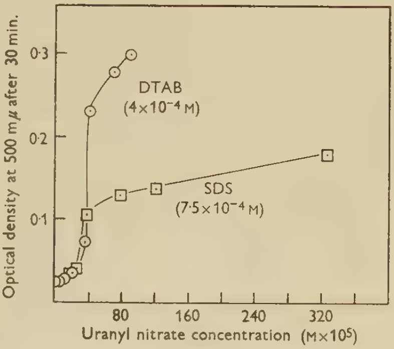

Fig. 6

Fig. 5. Lysis by sodium dodecyl sulphate of protoplasts pretreated with uranyl nitrate in M-sucrose + 0·05 M-NaCl solution. Concentration of SDS = $7{\cdot}5 \times 10^{-4}$ M. Concentrations of uranyl nitrate (M $\times 10^5$) given on curves.

Fig. 6. Lysis by detergents of protoplasts pretreated with uranyl nitrate. Optical density after 30 min. plotted as a function of uranyl nitrate concentration. Concentration of detergents indicated on curves.

DISCUSSION

Several factors have been considered important in governing the course of reaction of ionic detergents with the protoplast membrane of *Micrococcus lysodeikticus* (Gilby & Few, 1957*b*), including access of ions to the reactive sites in the membrane and the interactions between polar and between non-polar groups which give rise to binding and penetration. Comparison of the relative activities of cationic detergents in protoplast lysis (summarized in Table 1) indicates the importance of the nature of the substitution in the amines used, which all possess a C_{12} alkyl chain, and suggests that steric factors play an important role in their interaction with protoplasts. This is strikingly demonstrated by the substituted amine, DTAB, which has an activity relative to the unsubstituted amine of only 0·56. Inspection of molecular models of the detergents shows that the reactivity sequence DAC > DPB > DTAB is the opposite of the sequence of relative size of the ionizable head groups. Thus the most bulky group, $-N(CH_3)_3^+$, had the lowest lytic activity. This might be due to diminished ion-dipole forces (proportional to Z/r^3) involved in adsorption of the detergent, or possibly to restricted access to an internal adsorption site. Contrary to its actual ranking as the most active agent studied, DQB would, from size considerations, be expected to be less active than DPB. This enhanced activity seems likely to be due to the additional benzene ring of the quinolinium system which could cause a shift in the hydrophylic-hydrophobic balance of the molecule and thus alter its adsorption and penetrative powers. Analysis of the DQB used showed that impurities, which might have increased its activity, were absent (Few *et al.* 1958).

The protoplast membrane of *Micrococcus lysodeikticus* is a complex structure of protein, lipid and carbohydrate (Gilby *et al.* 1958). The lipid component consists chiefly of a complex phosphatidic acid and occupies an internal site in the membrane (Few *et al.* 1960). Such a phospholipid would provide suitable ionic groups for electrostatic interaction with cationic detergents. Furthermore, the net negative charge at the surface of the protoplast would favour the adsorption of cationic detergent ions. The inhibition of lysis by pretreatment with uranyl ions suggests that the important site of action in the protoplast membrane may be the phosphate groups of the membrane lipid. Some workers (e.g. Massart, 1952) have described a decrease in toxicity of surface-active cations by competing cations. Newton (1954) postulated that the extreme effectiveness of uranyl ions in protecting *Pseudomonas aeruginosa* from the lytic action of polymyxin suggested that the polymyxin-combining loci of that cell surface may be polyphosphates. The phosphate groups of the membrane lipid of *M. lysodeikticus* are particularly sensitive to uranyl ions (Few *et al.* 1960). As stressed previously (Gilby & Few, 1957*b*) DTAB is not bound by proteins such as bovine plasma albumin at concentrations which produce rapid lysis of protoplasts (Few, Ottewill & Parreira, 1955). It is therefore concluded that, in protoplast lysis, cationic detergents react with the phosphatidic acid lipid component of the protoplast membrane to disrupt its permeability properties. With detergents of similar hydrophilic-hydrophobic balance, the relative activity is controlled by the size of head group which can effect accessibility of the cationic detergent to the internal lipid site as well as the initial adsorption.

In previous work (Gilby & Few, 1957*b*) no distinction was made between the action of cationic and anionic detergents, both of which were assumed to lyse by essentially the same type of action. However, inspection of the lysis/time curves reveals a marked difference between the curves obtained with each type of detergent. Whereas the cationic detergents decreased the optical density to that of complete lysis at a rate depending on detergent concentration, the anionic detergents caused a comparatively rapid initial decrease to an optical density which subsequently changed only very slightly over many hours. No detailed picture of the mechanism of lysis has been established to explain the nature of the lysis/time curves for anionic detergents or the condition of the protoplasts which survive the initial rapid lysis. In the stationary state, when either more detergent or more protoplasts are added, the change in optical density is resumed and a new stationary value reached. This suggests that this behaviour reflects the distribution of susceptibility to anionic detergents among the protoplasts. However, it is possible to invoke other *ad hoc* ideas to explain such observations. For example it was postulated for erythrocytes by Love (1950) that anionic detergents exert a protective as well as a lytic action.

With suspensions of protoplast membranes of *Micrococcus lysodeikticus*, which are not subject to any osmotic stress, SDS had a precisely similar reaction curve to that with protoplasts, while DTAB appeared to have no action on the isolated membranes. Salton (1957) noted similar differences in the membrane residues visible by phase microscopy after lysis of protoplasts of *Bacillus megaterium* by detergents. Whereas SDS appeared completely to disperse the protoplast membranes, lysis of protoplasts of *B. megaterium* by cetyl trimethylammonium bromide was not accompanied by dispersion of the protoplast membranes. The differences in the form of the reaction curves of SDS and of DTAB with protoplast membranes of *M. lysodeikticus* suggest that a difference in action between the two types of detergent also exists in the reactions with isolated membranes, as well as in protoplast lysis. SDS interacts much more strongly with protein than does DTAB (Few *et al.* 1955). It therefore seems likely that the action of anionic detergents on the isolated protoplast membrane of *M. lysodeikticus* involves the protein moiety of the membrane and results in complete dissolution of the membrane.

Since in the protoplast-lysis experiments the concentration of membranes which might result from lysis was so low that the membranes could scarcely affect the measured optical densities, the identical nature of the protoplast and the membrane lysis curves suggests that a similar mechanism may operate in both protoplast lysis and membrane disintegration by anionic detergents. In this case, protoplast lysis would depend on the dissolution of protoplast membrane and not on the disruption of the membrane's impermeability to the stabilizing sucrose, then followed by osmotic explosion. On the other hand, it may be argued that the similarity of the concentration ranges for DTAB and for SDS in which uranyl nitrate inhibited protoplast lysis (Fig. 6) suggests a similar site of action for both detergents. It seems possible that SDS may react with both protein and lipid, or alternatively with a combination of both as lipoprotein. Van der Waals forces and ion-dipole interactions, for example with hydroxyl groups in the membrane lipid shown to be present by infrared spectroscopy (Gilby & Few, 1957*b*), might well be sufficient to cause adsorption and penetration of the lipid by SDS. A similar concept of two concurrent mechanisms which act in the haemolytic action of anionic detergents on red cells was suggested by Rideal & Taylor (1957). However, such an argument may not be valid for the lysis of protoplasts. It seems certain that anionic and cationic detergents act on different sites in the membrane or else act on a similar site but in different ways. From the evidence presented here it is concluded that the crucial action of anionic detergents in the lysis of protoplasts of *Micrococcus lysodeikticus* is with a protein component of the protoplast membrane, which might be involved in a lipoprotein complex; the direct result of this action is dissolution of the membrane. Cationic detergents, however, cause lysis as a secondary result of their action on the membrane lipid.

We thank Dr J. H. Schulman for helpful discussions and the Commonwealth Scientific and Industrial Reseach Organization, Australia, for a Postgraduate Overseas Studentship (A. R. G.). The late A. V. Few was a member of the scientific staff of the Medical Research Council.

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Lysis of Protoplasts of *Micrococcus lysodeikticus* by Alcohols

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(*Received 7 December* 1959)

SUMMARY

The time course of lysis of protoplasts of *Micrococcus lysodeikticus* by *n*-ethanol, *n*-propanol, *n*-butanol and *iso*amyl alcohol was studied spectrophotometrically. The relative activity for lysis increased with the molecular weight of the alcohol used. Equal degrees of lysis resulted at equal thermodynamic activities of the alcohols. The high lytic concentrations of ethanol and propanol produced side effects which obscured the lysis/time curves. *n*-Butanol showed time delays before the onset of lysis; the significance of this is discussed in terms of protoplast-membrane structure. Because of similarities with lysis by cationic detergents and with the extraction of serum lipids in the presence of alcohols, it is concluded that the alcohols tested acted on the lipid component of the protoplast membrane and that lysis resulted from the disruption of membrane permeability.

INTRODUCTION

As bactericidal agents alcohols are much less effective than ionic detergents; the optimum bactericidal concentration of ethanol is usually between 30 and 50 %. The germicidal action of the shorter-chain alcohols increases with molecular weight. These agents are believed to act by dissolving in the lipid layer of the cell membrane and breaking down its structure by solvent action (Danielli, 1951). Mitchell (1953) showed that, above a critical concentration, butanol caused release of intracellular phosphate from certain bacteria. Most of the work on the lytic action of alcohols has been on erythrocytes. From comparative studies of haemolysis and monolayer penetration, Pethica & Schulman (1953) concluded that alcohols haemolyse by a non-specific detergent process dependent upon a critical lowering of interfacial tension. While this applies at fairly rapid rates of haemolysis, the effect in general is more complicated since even very low concentrations of alcohols will eventually cause haemolysis (J. F. Hoffman, private communication). The adsorption of the alcohol is but the first step in a chain of processes. Pethica (1958) reviewed some possible mechanisms for the action of butanol on some membranes. Previous work (e.g. Salton, 1957; Gilby & Few, 1960) dealt with aspects of lysis of bacterial protoplasts by ionic detergents. As examples of non-ionic soluble compounds, we have now studied short chain alcohols. Protoplasts of *Micrococcus lysodeikticus* were studied during lysis by a series of primary *n*-alkyl chain alcohols and by one secondary alcohol. The technique used allowed the reagent to react directly with the protoplast membrane free from an intervening cell wall.

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METHODS

Compounds used. The *n* primary alcohols used were ethanol, propanol, butanol and pentanol. These compounds (initially of A.R. quality) were purified by fractional distillation and the major fractions were collected over a range of less than 0·1°. Preliminary experiments indicated that rigorous purity was essential as a small amount of higher molecular weight material could dominate the results. Some experiments were also performed with a fractionated sample of *iso*amyl alcohol.

Organisms used and protoplast formation. Micrococcus lysodeikticus (NCTC 2665) was grown on a beef tryptic-digest agar medium. The conditions in the controlled treatment with lysozyme to yield protoplasts and protoplast membranes were those of the preceding paper (Gilby & Few, 1960). Protoplasts were stabilized in M-sucrose +0·05 M-NaCl solution.

Spectrophotometric determination of lysis. The methods used were similar to those used with ionic detergents (Gilby & Few, 1960). The optical density at wavelength 500 mμ was measured during the reaction of protoplast suspension with alcohol at 22 ± 1°. The concentration of protoplasts corresponded to 0·5 mg. dry wt./ml., and the reaction medium (at pH 6·3 ± 0·1) contained M-sucrose +0·05 M-NaCl. The usual precautions were taken to avoid spurious damage to protoplasts. In the range of alcohol and protoplast concentrations used, the rate of lysis was independent of cell concentration.

RESULTS

The activity of alcohols in protoplast lysis

The optical density/time curves for lysis are given in Figs. 1–3. The concentrations of ethanol in Fig. 1 required to produce lysis are several times molar. These high concentrations of ethanol caused precipitation of intracellular constituents of protoplasts and, as a result, the final optical densities (*c.* 0·1) obtained on complete lysis by ethanol were larger than those obtained for lysis by higher alcohols. All concentrations of ethanol used gave rise to large increases in optical density in the initial stages of lysis. The lysis of protoplasts was more rapid at higher ethanol concentrations. With propanol (Fig. 2) the curves were broadly similar to those obtained with ethanol; the initial increases in optical density were smaller than the increases produced by ethanol. On complete lysis, the limiting value of optical density was near that corresponding to complete lysis by other means (osmotic explosion or ionic detergents). Thus the complication of precipitation with ethanol was not present in this range of propanol concentrations. In the results for butanol given in Fig. 3 the initial increases in optical density were very small. These curves suggested that there was a delay period before the onset of lysis and that the duration of the delay was decreased by increasing concentrations of butanol; there were no precipitation effects. With *n*-amyl alcohol it was impracticable to follow lysis since only extremely slow changes in optical density occurred, even at complete saturation. *Iso*amyl alcohol gave lysis/time curves similar in nature to those for butanol (Fig. 3).

The relative activities of the alcohols were compared in a manner similar to that used for ionic detergents (Gilby & Few, 1960). The decrease in optical density after 30 min. treatment has been plotted in Fig. 4 as a function of detergent concentration.

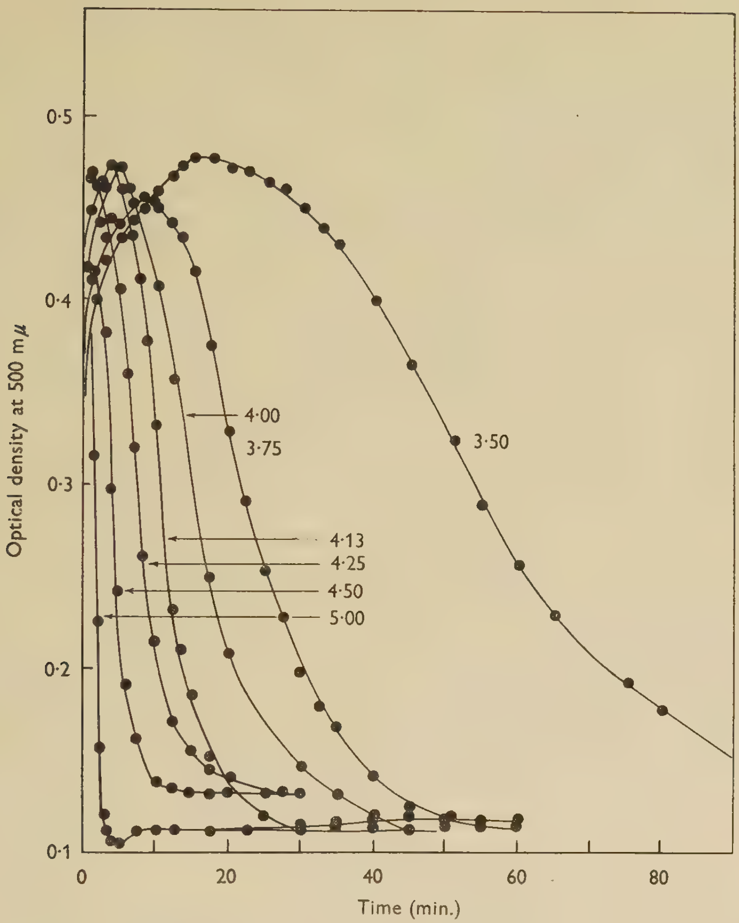

Fig. 1. Lysis of protoplasts of *Micrococcus lysodeikticus* by ethanol in M-sucrose + 0·05 M-NaCl at concentrations of ethanol (M) given on curves.

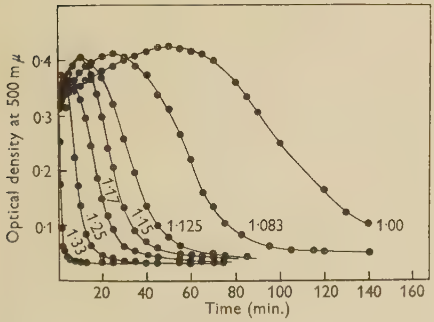

Fig. 2

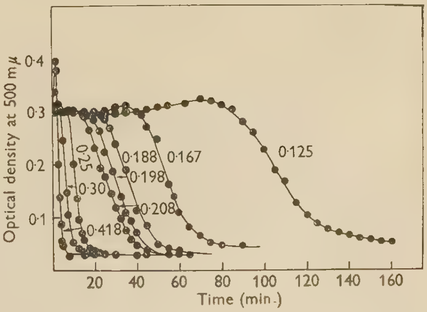

Fig. 3

Fig. 2. Lysis of protoplasts by *n*-propanol in M-sucrose + 0·05 M-NaCl at concentrations of *n*-propanol (M) given on curves.

Fig. 3. Lysis of protoplasts by *n*-butanol in M-sucrose + 0·05 M-NaCl at concentrations of *n*-butanol (M) given on curves.

As previously, the protoplast lytic concentration was defined as that concentration of alcohol which in 30 min. decreased the optical density of protoplast suspension by 90 % of the loss observed on complete lysis. The curves in Fig. 4 for butanol and propanol are very steep and the determination of the protoplast lytic concentration was straightforward. With ethanol, the slope was greater, partly because of precipitation and the influences of the large initial increases in optical density. Extrapolation to the 90 % line for other alcohols was not used. The definition was applied literally and the experimentally found specific 90 % line was used to derive the protoplast lytic concentration as indicated.

The protoplast lytic concentrations of the alcohols and their activities relative to ethanol are given in Table 1. The lytic activity increased greatly with increase in chain length. A further comparison of the data was made by calculating the thermodynamic activity of each alcohol in the aqueous phase; the thermodynamic activity is a general expression of the 'effective' concentration of the alcohol. This differs from the actual concentration because of interactions between alcohol and solvent. The thermodynamic activities in Table 1 are only approximate because the values of the thermodynamic activity-coefficient used apply to solution in water, whereas the alcohols used here were dissolved in M-sucrose +0·05 M-NaCl solution. In the range of concentrations used, concentrations were substituted for mol fractions without serious inaccuracy in calculating thermodynamic activity. The values of thermodynamic activity calculated in this manner for the four alcohols are approximately equal. Considering the assumptions involved, the agreement is good. Thus, for the lysis of protoplasts by the aliphatic alcohols used, equal degrees of lysis resulted at equal thermodynamic activities of alcohol.

Table 1. *Protoplast-lytic concentrations and thermodynamic data for alcohols*

Substance	Protoplast lytic concn. (M)	Activity relative to ethanol	W*	Thermodynamic activity (A_W)
Ethanol	4·02	1·0	3·69	14·8
1-Propanol	1·18	3·4	11·6	13·5
1-Butanol	0·24	16·8	51·0	12·2
2-Pentanol	0·08	50·0	191	15·3

* W is thermodynamic activity coefficient in water of the alcohol at the protoplast-lytic concentration (from Butler, Thomson & Maclennan, 1933, and from Butler, 1937).

The kinetics of lysis of protoplasts of Micrococcus lysodeikticus

The two general types of data used in the study of haemolysis are: (i) % haemolysis/time curves; (ii) time/dilution curves (Ponder, 1948). The lysis/time curves in Figs. 1–3 for protoplast lysis are of the former type. Because of the complications with ethanol and propanol, attention will be confined here to lysis with butanol. The curves in Fig. 3 appear to fall into two groups. The curves at butanol concentrations of 0·25 M and higher form a similar group whilst those at more dilute concentrations exhibit, after the initial delay period, a comparable course of reaction. By multiplying the time scale of the 0·208 M curve by an appropriate factor, the curves for smaller dilutions can be accurately reproduced. A similar division of the kind of lysis into two concentration ranges is achieved by plotting delay times against butanol concentrations (Fig. 5). Here again the shape of the curves suggests that a change in character may occur at a butanol concentration of *c.* 0·2 M. Other empirical plots of functions derived from the data reinforce this conclusion. As an example of the second type of treatment of data, a typical time/concentration curve which corresponds to *c.* 80 % lysis by *n*-butanol is given in Fig. 5. Whilst kinetic equations can be derived to describe such curves, these equations contain constants of which the physical nature is obscure. Such formal equations would contribute little to an understanding of the principles involved.

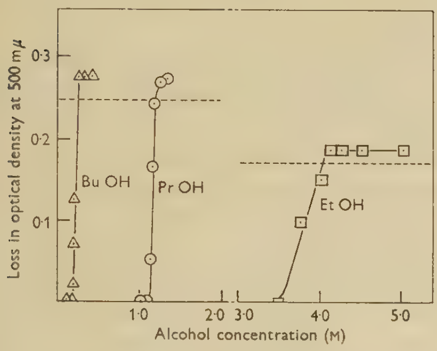

Fig. 4

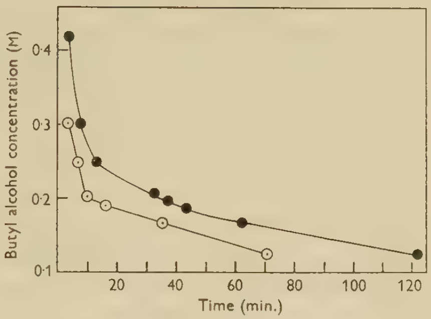

Fig. 5

Fig. 4. Decrease in optical density of suspensions of protoplasts of *M. lysodeikticus* after 30 min. lysis plotted as a function of alcohol concentration. Broken lines indicate 90 % lysis.

Fig. 5. Time/concentration curves for lysis of protoplasts of *M. lysodeikticus* by *n*-butanol in M-sucrose + 0·05 M-NaCl solution. ● = Time to reach optical density 0·1, ⊙ = delay times before onset of lysis.

DISCUSSION

The lysis of protoplasts of *Micrococcus lysodeikticus* by alcohols bears some resemblance to the phenomenon of narcosis. At equilibrium the activity of alcohol must be the same in all phases and the activity in the external medium may be used to indicate the activity of alcohol in the protoplast membrane. If the activity coefficients of the alcohols in the sensitive site in the protoplast membrane are approximately the same (as they are in benzene; Butler & Harrower, 1937), then the data in Table 1 indicate that equal concentrations of alcohol in the membrane will produce equal degrees of lysis. Brink & Posternak (1948) suggested an empirical rule for narcosis which was analogous to this. Mullins (1954) argued that a better generalization would be that there were equal degrees of narcosis with equal volume fractions of narcotic in the membrane. In the present instance, when the thermodynamic activities at the protoplast lytic concentrations are multiplied by the molar volumes of the alcohols, the constancy of the products is slightly worse than that of the activities. However, there is no *a priori* reason why lysis and narcosis should have identical mechanisms. Indeed, narcosis has been suggested to result either from the prevention of the passage through the membrane of particular molecules or ions, depending on the degrees of occlusion by the narcotic, or else by action on intracellular lipids. Protoplast lysis, on the other hand, follows a breakdown in the impermeability of the protoplast membrane to solutes which normally protect the cell from osmotic rupture. Other results which present features similar to those of protoplast lysis have been encountered in studies on the extraction of lipids from serum by di-ethyl ether in the presence of alcohols. Machebouf & Sandor (1932) obtained curves for the extraction of lipid which are similar to the protoplast lysis/time curves, both in shape and in the occurrence of a delay period. The essential feature of their explanation was that di-ethyl ether cannot penetrate and dissolve the lipid until the alcohol has brought about far-reaching changes in organization of the lipid. Although Delaye (1935) found that butanol was not markedly more effective than ethanol, this could be due to partition between the ether solvent and the aqueous phase masking the greater activity of the butanol.

The alcohols interact with the protoplast membrane in different ways depending on the concentrations involved. The increases in optical density noted in the early stages of lysis with ethanol and propanol are greater than any observed to be produced by osmotic changes in volume (Gilby & Few, 1959). These observations are more likely to result from changes in refractive index at the protoplast/medium interface by denaturation or dehydration of the outer protein carbohydrate layer of the protoplast shown to be present by electrophoresis (Few, Gilby & Seaman, 1960). The effect is much less marked with butanol which has lower lytic concentrations. Uranyl nitrate caused inhibition of lysis over a similar range of concentrations to that which inhibited lysis by ionic detergents (Gilby & Few, 1960). Detailed results for this effect have not been presented because replication was poor. Furthermore, protoplast membrane suspensions treated with *n*-butanol at concentrations over the range which produced lysis showed no change during 12 hr. In this way the action of the alcohol resembles that of cationic detergents and indicates that the protein matrix of the membrane is not broken down. From these results and the strong similarities to the serum–lipid extraction discussed above, it is concluded that the alcohols lyse protoplasts of *Micrococcus lysodeikticus* by their action on the lipid component of the protoplast membrane. Although the specific type of action on the lipid is uncertain, *n*-butanol may function by virtue of its property as a breaker of hydrogen bonds (Pethica, 1958). The shape of the butanol lysis/time curves can then be explained as follows. The delay period represents the time taken by the alcohol at a given concentration to penetrate and disrupt the organization of the protoplast-membrane lipid. Lysis then results from the changed permeability of the membrane and consequent loss of protection against osmotic explosion. The sigmoid shape of the curve, once lysis has begun, reflects the distribution of resistance to lysis of the protoplasts.

Attempts have been made to account quantitatively for the experimentally found delay periods with *n*-butanol, using models based on the above ideas. One such model in accord with data on the structure of the protoplast membrane is that the alcohol must diffuse through: (i) an initial barrier at the protein/aqueous interface in the protoplast surface; (ii) a relatively thick 'sandwich' layer of protein-carbohydrate; (iii) a second barrier at the protein/lipid interface. The equations based on this and other models to express the time taken for an alcohol to diffuse through different layers and to reach a critical concentration all suffer from the same fault in that they give delay times which do not increase fast enough as the concentration is decreased. Should the diffusion properties in the outer layers be time-dependent, this would complicate the model considerably. A successful quantitative account of the course of protoplast lysis must await a more detailed understanding of the events which precede and accompany the lysis. Evidence from other cells suggests that the penetration of the agent which causes lesion in the membrane is fast relative to the subsequent changes which result in lysis (e.g. volume changes in red cells; Parpart & Green, 1951). Should this be so with protoplasts, it might explain the failure of the deductions from simple diffusion models. It is suggested that further studies on protoplasts in the presence of butanol during the pre-lytic delay period, e.g. investigations on the leakage of intracellular constituents and interrupted lysis, may be instructive.

We thank Dr J. H. Schulman for his interest in this work and the Commonwealth Scientific and Industrial Research Organization (Australia) for a Postgraduate Overseas Studentship to one of us (A. R. G.). The late A. V. Few was a member of the scientific staff of the Medical Research Council.

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The Additive Effect of Glycine and other Amino Acids on the Induction of the L-Phase of Group A β-Haemolytic Streptococci by Penicillin and D-Cycloserine

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SUMMARY

The ability of glycine to induce L-phase of bacteria did not apply to group A β-haemolytic streptococci. In the presence of penicillin, glycine in a bacterial inhibitory concentration had a notably additive L-phase inducing property for streptococci. Reasons are given to support the view that this activity of glycine is limited to the induction of L-phase growth. A sharp increase in the yield of L-phase colonies was also observed when serine, threonine, methionine or alanine were tested under similar conditions. When the effectiveness of the racemate and both the isomers of these amino acids was compared, the D-isomer was the active substance in most cases. In one strain, the L-inducing and bacterial inhibitory action of the racemate and both isomers of methionine was tested simultaneously. The yield of L-phase colonies was found to be inversely proportional to the effect of the different stereoisomers on bacterial growth.

D-Cycloserine had a weak L-inducing effect on group A streptococci, but the combined action of D-cycloserine and glycine or DL-serine was equal to that obtained with penicillin and the same amino acids. Propagation of these L-phase organisms was possible in the presence of 1% (w/v) glycine and within a narrow range of concentrations of D-cycloserine. The additive effect of the amino acids cited towards penicillin and D-cycloserine for the induction of L-phase structures can be explained by the antibiotic property of high concentrations of glycine and some of the D-amino acids.

INTRODUCTION

The induction of the L-phase of *Streptobacillus moniliformis* by penicillin was first demonstrated by Pierce (1942). Since then, this substance has been used for the induction of the L-phase of many types of Gram-positive and Gram-negative bacteria. In addition to penicillin, a certain number of other agents appear to have L-inducing properties. Gordon & Gordon (1943) described a peculiar action of glycine on organisms of the genus *Vibrio*. After the addition of 0·25% to 1·5% (w/v) glycine, the bacteria became swollen and enlarged. At 2% this phenomenon ceased as a result of total growth inhibition. In the high range of active concentrations very few ordinary bacillary types were seen. The picture was mainly that of large circular forms, wherein signs of autolysis were also present. Maculla & Cowles (1948) showed that high concentrations of glycine caused lysis of different organisms, whereas organisms adapted to high concentrations of this substance (Gordon, Hall & Stickland, 1949) were insensitive to this action; their growth properties remained undisturbed. Dienes & Weinberger (1951) obtained L-phases of bacteria from several species by using glycine, but penicillin was effective with many more species than was glycine. Dienes & Zamecnik (1952) examined other amino acids for this effect. These substances were tested on strains of *Salmonella typhimurium* and *Haemophilus influenzae*. Both organisms produced L-colonies abundantly in the presence of glycine or penicillin. Positive results were also obtained with a few other amino acids; L-colonies developed with concentrations varying between 0·5–3·0 % (w/v) glycine, DL-methionine, L-phenylalanine and L-tryptophan. Glycine was the most effective amino acid.

Although the problem of the possible identity between L-phase growth and protoplasts will not be discussed here, these structures show enough similarities to justify mutual exchange of methods for isolation purposes. Welsch (1958) reported on protoplast formation with *Escherichia coli* exposed to pencillin or glycine as well as serine, alanine and α-aminobutyric acid. Lark & Lark (1958) obtained protoplast-like structures from *Alkaligenes faecalis* in the presence of certain D-amino acids. As cycloserine was reported by Ciak & Hahn (1959) to induce protoplast formation in *Escherichia coli*, the study of this compound was also included in the present work. The use of the term protoplast by these authors may now need revision in the light of the suggestions by Brenner *et al.* (1958). Some of these forms could probably be classified as spheroplasts according to McQuillen (1959).

Having established that glycine alone was ineffective in any concentration between 0·25 and 3 % in inducing the L-phase growth of group A β-haemolytic streptococci, the effect of this substance was examined when incorporated in a medium suitable for growing L-structures of this species. It appeared then that glycine, and some other amino acids, either as the racemate, or especially as the D-isomer, markedly increased the yield of the L-phase colonies in the presence of penicillin or D-cycloserine.

METHODS

Organisms. All strains tested belonged to group A β-haemolytic streptococci. Strains AED (type 12), GL 8 (type 19), ADA (type 14), were obtained from Dr L. Dienes (Boston, U.S.A.), the other strains B1 (type 1), B4 (type 1), B6 (type *11*.27.44), B7 (type 12), B8 (type *11*.27.44), B9 (type 12), B14 (type 3), B17 (type 22), were of local origin. All strains except GL 8 and ADA were typed with the agglutination reaction.

Medium. The medium consisted of a tryptic digest of beef heart infusion with 1·3 % (w/v) agar. Before pouring the plates, horse serum previously inactivated at 56° for 30 min. and sodium chloride were added (final concentration 10 % and 0·6 M respectively). The final volume in each plate was 15 ml. This medium plus penicillin will be referred to as the basal medium.

Amino acids. The racemates and single isomers of serine, threonine, alanine and methionine (Hoffmann-La Roche), and glycine were made up in the maximum concentration. After autoclaving at 15 lb./sq.in. for 15 min. (121°), amounts were added to the basal medium to final percentages ranging from 0·25 to 1·5 % (w/v) of the different substances tested.

Antibiotics. The D-cycloserine used was the synthetic product (Hoffmann-La Roche). A solution containing 100 mg./ml. was sterilized through a glass filter. A commercial preparation of the potassium salt of penicillin was used in a solution of 200,000 units/ml.

Induction of the L-phase. The technique used for isolation of the L-phase of group A β-haemolytic streptococci was that described by Sharp (1954). The plates were inoculated with a 14–16 hr. culture grown in glucose broth. After incubation for 1·5 hr. at 37°, three small troughs were cut in the centre of the agar and a quantity of the solutions equivalent to 400 units penicillin or 3·0 mg. D-cycloserine, was deposited in each trough. The plates were then incubated for a week at 37° semi-anaerobically by the Fortner (1928) method.

Dry weights. The evaluation of growth of the unmodified streptococcus strain B1 in the presence of different concentrations of glycine was performed by measuring dry weights of bacteria after 48 hr. incubation in the same medium without the addition of agar, salt and penicillin. In this case the evaluation of bacterial growth by measuring dry weights was the only reliable method, because glycine gives rise to inhomogeneous granular suspensions (Gordon & Gordon, 1943).

Turbidimetry. When the streptococcus strain AED was tested for the effect of the racemate or the single isomers of methionine, it showed homogeneous suspensions after growth. This permitted the evaluation of growth variations caused by the three substances as turbidity readings, using a Unicam Spectrophotometer at a wavelength of 550 mμ. The medium consisted of Heart Brain Infusion (Difco), enriched with 10% (v/v) horse serum without added salt or penicillin.

Minimal inhibitory concentrations. The inhibitory effect of D-cycloserine on streptococcus strain AED was assessed by measuring the turbidities of the growth in a conventional dilution series ranging from 100 to 500 μg. D-cycloserine in glucose broth. The turbidities were read at 550 mμ after incubation for 24 hr. at 37°.

Explanation of symbols. The number of L-phase colonies in the basal medium was verified between 4–6 times for all strains, and ranged from just a few to several hundreds, depending on the strain tested. Reproducibility within each strain was satisfactory for the purposes of this study. The counting was done by means of a stereomicroscope, and was practicable up to 600 colonies/plate. Only the highest figure of each control series is recorded, and this figure is given between brackets. When the number of L-phase colonies exceeded 600 (which never occurred in the control series) this was registered with multiples of the + sign. The estimated number of L-phase colonies scored with ++ was between 600 and 1000; with +++ between 1000 and 4000; with ++++ over 4000 colonies.

RESULTS

All efforts to grow the L-phase of group A β-haemolytic streptococci with glycine as an inducing agent failed but it was found that the combined action of glycine and penicillin considerably enhanced the number of L-phase colonies as compared with that obtained with penicillin alone (Table 1). The B1 strain tested under these conditions never gave more than 50 L-phase colonies in many controls, whereas about 750 colonies were obtained when the medium contained 1·25% (w/v) glycine. The L-inducing action of glycine, up to 1·25%, was roughly inversely proportional

Table 1. *Influence of increasing concentrations of glycine on the yield of L-phase colonies from a strain of group A β-haemolytic streptococci on the basal medium, and on dry weights of the coccal form of the same strain grown in an identical medium without the addition of agar, penicillin and salt*

Strain	Glycine concentration % (w/v)	No. of L-phase colonies	Dry weights of bacteria in mg./ml.
B 1	Control	50	0·348
	0·5	28	0·323
	0·75	150	0·318
	1·0	500	0·263
	1·25	750	0·116
	1·5	200	0·063

The number of L-phase colonies obtained in many experiments with the B 1 strain on the basal medium never exceeded 50.

Table 2. *Effect of the addition of varying concentrations of glycine or* DL-*serine to the basal medium on the yield of L-phase colonies in eight strains of group A β-haemolytic streptococci*

Strain ...	B1	B4	B6	B7
Control ...	+ (50)	+ (196)	+ (66)	+ (68)
		Glycine		
0·75 %	+ (142)	+ (400)	+ (75)	+ +
1 %	+ (500)	+ + +	+ (150)	+ + +
1·25 %	+ +	+ + + +	+ (300)	+ + +
1·5 %	+ (200)	+ + +	+ +	+ + + +
1·75 %	nt	+ +	+ +	+ + +
		DL-Serine		
0·75 %	nt	None	None	+ (350)
1·0 %	+ +	None	+ (68)	+ (400)
1·25 %	+ + +	None	None	+ (165)
1·5 %	+ + +	None	None	+ + +
1·75 %	nt	None	None	+ +

Strain ...	B8	B9	B14	B17
Control ...	+ (1)	+ (27)	+ (67)	+ (101)
		Glycine		
0·75 %	+ + +	None	+ + +	+ +
1·0 %	+ +	+ +	+ (304)	+ + +
1·25 %	+ +	+ +	+ +	+ (106)
1·5 %	+ + +	+ + +	nt	+ + +
1·75 %	+ + + +	+ + +	nt	+ + + +
		DL-Serine		
0·75 %	+ (74)	+ (5)	+ (16)	+ (124)
1·0 %	+ +	+ (22)	None	+ (184)
1·25 %	+ (47)	+ (110)	+ +	+ (53)
1·5 %	+ +	+ +	nt	None
1·75 %	+ + +	+ + +	nt	None

Figures between parentheses are exact numbers of L-phase colonies counted in the plate. The number of control plates for all strains varied from 4 to 6. Only the highest figure of these is given here each time. Evaluation of colony numbers is expressed as: + = 0–600, + + = 600–1000, + + + = 1000–4000, + + + + = 4000 or more colonies. nt = not tested.

to its growth inhibitory effect, according to the dry weights of organisms harvested from media containing the different concentrations of amino acid.

To examine the effect of other amino acids DL-serine (structurally related to glycine) was selected. As early experiments gave results comparable with those obtained with glycine, eight strains were tested simultaneously for the effect of glycine or DL-serine (Table 2). The concentrations of the amino acids used were between 0·75 and 1·75% (w/v), the highest ones being tested only as far as necessary. High yields of L-phase colonies were obtained in all cases with glycine at 1·25—1·5%. With DL-serine, the results were equally good with 5 strains (B1, B7, B8 and B14) out of 8. Except for the B1 strain, the effective concentration of this amino acid was found at 1·5–1·75%. Three strains (B4, B6, B17) on the other hand, remained unaffected even when exposed to high concentrations of DL-serine.

Possible differences in activity as between the individual isomers of DL-serine and the racemate were then considered. Four strains (B6, AED, GL8 and ADA) were tested in this respect, at concentrations varying between 0·25 and 1·5% (Table 3). It appeared that the additive L-phase inducing property of DL-serine toward penicillin, could be attributed in all the strains to the D-isomer. The L-isomer on the other hand, did not prevent the formation of L-phase colonies, even in the high range of concentrations in two out of the four strains (B6 and AED). It is to be noted that the B6 strain, which showed no influence of DL-serine at all, was favourably affected even by a concentration as low as 0·25% of D-serine. The increase of the L-phase growth of strain B6 by D-serine is shown in Plate 1.

The experiments were repeated with the same strains under similar conditions but with the chemically related threonine in place of serine. The results (Table 4)

Table 3. *Effect of the addition of varying concentrations of the racemate and both the isomers of serine on the yield of L-phase colonies in four strains of group A β-haemolytic streptococci*

Strain		DL-Serine	L-Serine	D-Serine
B6	Control	+ (66)	+ (66)	+ (66)
	0·25%	None	+ (1)	+++
	0·5%	None	None	+++
	1·0%	None	None	++++
	1·5%	None	+ (47)	++++
AED	Control	+ (600)	+ (600)	+ (600)
	0·25%	+++	+ (180)	+++
	0·5%	+++	+ (120)	+++
	1·0%	++++	+ (68)	++++
	1·5%	++++	+ (78)	++++
GL 8	Control	+ (174)	+ (174)	+ (174)
	0·25%	+ (3)	None	+++
	0·5%	+ (17)	None	+++
	1·0%	+ (53)	None	++++
	1·5%	+++	None	++++
ADA	Control	+ (400)	+ (400)	+ (400)
	0·25%	+ (8)	None	++
	0·5%	+ (25)	None	+++
	1·0%	+ (89)	None	++++
	1·5%	++	None	+++

The explanation of the symbols is given in Table 2.

with threonine were on the whole similar to those obtained with serine, but the number of L-colonies of the B6 strain was increased by both the racemate and the single D-isomer. The L-isomer moderately enhanced the number of L-phase colonies in the AED strain, but not in the three remaining strains.

Table 4. *Effect of the addition of varying concentrations of the racemate and both the isomers of threonine on the yield of L-phase colonies in four strains of group A β-haemolytic streptococci*

Strain		DL-Threonine	L-Threonine	D-Threonine
B6	Control	+ (66)	+ (66)	+ (66)
	0·25 %	nt	nt	nt
	0·5 %	++	+ (67)	++
	1·0 %	+++	+ (55)	+++
	1·5 %	+++	+ (175)	+++
AED	Control	+ (600)	+ (600)	+ (600)
	0·25 %	+++	++	+++
	0·5 %	++	++	++
	1·0 %	++	++	+++
	1·5 %	++	++	++++
GL 8	Control	+ (174)	+ (174)	+ (174)
	0·25 %	++	+ (41)	++
	0·5 %	++	+ (33)	+++
	1·0 %	++	+ (20)	+++
	1·5 %	+++	+ (87)	++++
ADA	Control	+ (400)	+ (400)	+ (400)
	0·25 %	+ (110)	+ (400)	+++
	0·5 %	++	+ (175)	+++
	1·0 %	+++	+ (50)	++++
	1·5 %	++	+ (80)	+++

The explanation of the symbols is given in Table 2.

Of the amino acids found in the cell walls of group A streptococci, namely alanine, lysine and glutamic acid (Salton, 1953; Cummins & Harris, 1956; Hayashi & Barkulis, 1959) only alanine was tested as the racemate and the isomers with four strains. Though a positive result was detected, the results (Table 5) were on the whole less marked than the previous ones. Strain B6 did not respond to the addition of DL-alanine; strain AED behaved as it did in the presence of L-threonine, i.e. a moderate increase in the number of L-phase colonies, and strain ADA reacted exclusively but weakly to the presence of the D-isomer.

The AED strain was tested with methionine, as the L-inducing property of its racemate was mentioned by Dienes & Zamecnik (1952). The strain was also exposed to concentrations (0·125–1 %, w/v) of the racemate and the isomers in a liquid medium without addition of penicillin and salt, in order to evaluate the effect of the various configurations of the amino acid on the growth of the organisms in the coccal form. The figures for L-phase colonies are given in Table 6, and the turbidimetric data evaluating bacterial growth in Fig. 1. It can be seen that in the presence of the racemate and the D-isomer of methionine, high numbers of L-phase colonies arose, whereas only a few grew in the presence of the L-enantiomorph. On the other hand, the results with bacterial growth point to a reversed situation, i.e. the racemate and the D-isomer caused moderate and strong inhibition of growth

Table 5. *Effect of the addition of varying concentrations of the racemate and both the isomers of alanine on the yield of L-phase colonies in four strains of group A β-haemolytic streptococci*

Strain		DL-Alanine	L-Alanine	D-Alanine
B6	Control	+ (66)	+ (66)	+ (66)
	0·25 %	+ (130)	+ (130)	+ (192)
	0·5 %	+ (120)	+ (100)	+ (140)
	1·0 %	+ (210)	+ (58)	+ + +
	1·5 %	+ (15)	+ (7)	+ + +
AED	Control	+ (600)	+ (600)	+ (600)
	0·25 %	+ +	+ (450)	+ + +
	0·5 %	+ + +	+ + +	+ + +
	1·0 %	+ + +	+ + +	+ + +
	1·5 %	+ +	+ + +	+ + + +
GL8	Control	+ (174)	+ (174)	+ (174)
	0·25 %	+ (48)	+ (45)	+ (67)
	0·5 %	+ (111)	+ (30)	+ (120)
	1·0 %	+ (524)	+ (36)	+ (380)
	1·5 %	+ (75)	+ (46)	+ + +
ADA	Control	+ (400)	+ (400)	+ (400)
	0·25 %	+ (10)	+ (7)	+ (480)
	0·5 %	+ (24)	+ (8)	+ (15)
	1·0 %	+ (68)	+ (9)	+ (202)
	1·5 %	+ (67)	+ (2)	+ (54)

The explanation of the symbols is given in Table 2.

respectively whereas the L-enantiomorph had little effect even when present in concentrations as high as 1 %.

The next question concerned the influence of an amino acid with L-inducing property on the growth of already existing L-colonies. It appeared that replating L-structures induced in the presence of glycine for example, was fully practicable and these structures preserved their viability. But the number of L-phase colonies growing from equal amounts of diluted ground agar containing L-colony particles and replated in pour plates was decidedly higher in plates containing the basal medium (1000 units penicillin/ml.) without added glycine as compared with others containing even moderate concentrations of glycine. It was concluded that the action of glycine, and probably of other amino acids, had no growth-promoting effect on the L-phase, but was restricted to its L-inducing effect on the cocci, as might be expected because of the high concentrations of the substances required to produce the phenomenon.

In view of the work of Ciak & Hahn (1959), the possibility was considered of inducing the L-phase of group A β-haemolytic streptococci with D-cycloserine. It was found when growing the AED strain on the basal medium that the mere replacement of penicillin by D-cycloserine (3 mg./trough) allowed the growth of 5–10 small L-phase colonies in the zone of inhibition. When, however, glycine or DL-serine was added to the medium, the yield of L-phase colonies of the same strain was comparable with that obtained normally with penicillin and these amino acids. The effect of D-cycloserine alone was thus inferior to the action of penicillin; but upon addition of glycine or DL-serine D-cycloserine showed comparable activity to penicillin. Penicillin has hitherto been the sole antibiotic found suitable for the isolation of L-structures. Now it is shown that D-cycloserine can produce the same phenomenon, especially in the presence of some amino acids. It also appeared feasible though difficult to propagate the L-phase colonies obtained with D-cycloserine to a maximum of 1000 μg./ml. The minimal inhibitory concentration for the coccal form of the AED strain was assessed at 250 μg. D-cycloserine/ml., which corresponds to the concentration found by Ciak & Hahn (1959) for the group A Richards strain of streptococcus.

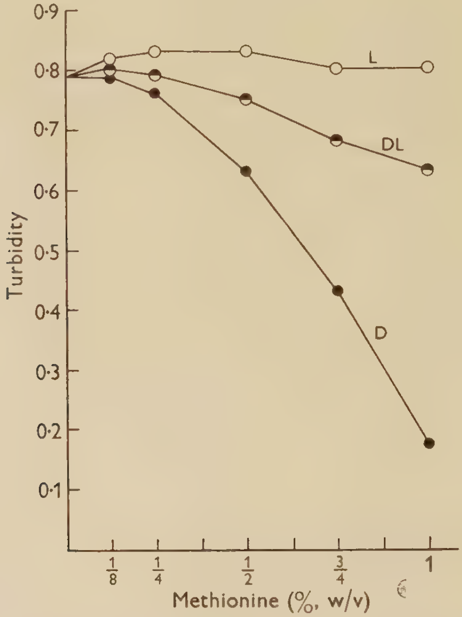

Fig. 1. Influence of the racemate and both the isomers of methionine on the growth of group A β-haemolytic streptococci. Equal inocula of an 18 hr. broth culture of the AED strain were grown in Heart Brain Infusion (Difco) enriched with 10 % (v/v) horse serum. Growth variations caused by different concentrations of the three substances were evaluated as turbidity readings at maximal growth, with a Unicam Spectrophotometer at a wavelength of 550 mμ. Maximal growth of the cells in this rich medium was obtained after about 14 hr. incubation at 37°. ◓——◓, DL-Methionine; ○——○, L-methionine; ●——●, D-methionine.

Table 6. *Effect of the addition of varying concentrations of the racemate and both the isomers of methionine on the yield of L-phase colonies in one strain of group A β-haemolytic streptococci*

Strain		DL-Methionine	L-Methionine	D-Methionine
AED	Control	+ (600)	+ (600)	+ (600)
	0·25 %	+ +	+ (1)	+ + +
	0·5 %	+ + +	+ (1)	+ + + +
	1·0 %	+ + +	+ (4)	+ + +
	1·5 %	+ + + +	+ (3)	+ + +

The explanation of the symbols is given in Table 2.

DISCUSSION

The L-phase inducing property of glycine as described by Dienes & Zamecnik (1952) with *Haemophilus influenzae* and *Salmonella typhimurium*, does not seem to apply to group A β-haemolytic streptococci. Nevertheless, glycine cannot be considered entirely devoid of this property with group A streptococci, as the number of L-phase colonies increased about twenty-fold or more with all the strains examined, when glycine was added to the basal medium (containing penicillin). The same additive principle was found to be true for serine, threonine, methionine and alanine, and it was further shown that the D-isomers of these amino acids were responsible for this effect. It is not unlikely that other amino acids will be shown active in this respect, although the low solubility of many of them severely limits further experiments, as high concentrations in general were required to elicit the phenomenon. However, the fact that in most cases the D-isomers were active in the lowest concentrations tested must be considered an advantage.

Dienes & Zamecnik (1952) mentioned that the L-inducing capacity of amino acids was always found at bacterial inhibitory concentrations and this finding could be confirmed in our studies. When optical isomers were tested the bacterial growth was not influenced by the L-enantiomorph nor did the L-phase colonies arise in its presence. The yield of L-phase colonies, however, increased when bacterial growth was inhibited by the presence of either the racemate or the D-isomer. The streptococcus strain AED formed an exception and showed in addition a moderate increase of L-phase colonies in the presence of L-threonine and L-alanine. If the view is accepted that a degree of bacterial inhibition by amino acids favours L-type growth induction it could be of value to examine whether the inhibitory influence of the three configurations of several amino acids on the growth of different streptococcal strains is liable to variability. This would give a ready explanation for deviations from the general rule such as were found in the AED strain.

References to the growth-inhibitory effect of amino acids in high concentrations are scarce in the literature. Fling & Fox (1945) examined the inhibitory action of the racemate and both the isomers of valine, leucine and alanine at 2% (w/v) with *Lactobacillus arabinosus*. The effect on growth exhibited the same pattern as was found here with methionine in group A β-haemolytic streptococci. D-Alanine however, did not inhibit growth. Gordon & McLeod (1926) also reported that D-alanine did not influence growth in different species at various concentrations up to 1%. Though these findings seem in contradiction in the first instance with the foregoing, it is possible that the deviating pattern for alanine has some, as yet unknown relation, to the quantitatively exceptional position which this amino acid occupies in streptococcus. In accordance with this hypothesis, the increase of L-phase structures obtained with alanine was found to be less striking, and perhaps associated with higher concentrations than in the case of the other amino acids tested.

Attention was especially focused on D-amino acids, since they were identified as fractions of peptides with antibiotic property. In discussing this subject, Work (1948) gave plausible reasons for the difference in antibiotic activity of D-amino acids as a part of such peptides and as pure substances. As already stated, some D-amino acids can in fact exhibit antibiotic activity provided that their concentration has been raised sufficiently. The activity of the amino acids in the present experiments can also be related exclusively to an enhancement of the action of the antibiotic present, and the fact that growth of L-structures arises at concentrations which inhibit bacterial growth denotes the relative insensitivity of the L-phase structures for these substances compared with the coccal form. The possible practical implications of these findings will not be discussed here. That the insensitivity of the L-phase structures to glycine is relative could be shown by the higher yields of subcultures in the basal medium as compared with propagation in the presence of additional glycine.

L-phase colonies and protoplasts have been defined as cell structures which are devoid of their cell walls. This view has received confirmation by chemical and serological studies of group A β-haemolytic streptococci (Sharp, Hijmans & Dienes, 1957) and other species as reviewed by Roberts, McQuillen & Roberts (1959). The accumulation of a N-acetylamino sugar precursor of cell walls of *Staphylococcus aureus* incubated with penicillin was reported by Park & Strominger (1957), and explained as a consequence of deficient cell-wall formation following the action of the antibiotic. From this, L-forms and spheroplasts can be also defined as biologically independent cell structures which arise by selective inhibition of cell wall synthesis. Until now the degree of selectivity exhibited by penicillin has not been equalled.

If other substances can provoke the accumulation of compounds similar to those isolated by Park & Strominger (1957), the possibility of inducing L-structures and spheroplasts with these arises, provided they are not toxic to the remaining structures. Accumulation of the cell-wall precursor compound was demonstrated with, in addition to penicillin, bacitracin, glycine, and oxytetracycline by Park (1958), and with D-cycloserine by Ciak & Hahn (1959).

It is very likely that the reverse will also be true, i.e. substances that can give rise to L-phase structures and spheroplasts will appear to accumulate the cell-wall precursor. Therefore the isolation of 'protoplasts' with several amino acids by Welsch (1958), with D-amino acids by Lark & Lark (1958) and the present findings on L-phase structures, strongly suggest that several D-amino acids can be added to the substances already mentioned.

In this connexion, it seems worth mentioning that while the L-inducing property of D-cycloserine was very weak and that of glycine absent, the substances in combination induced a great many fully grown L-structures. It is suggested that the morphological effects resulting from this additive phenomenon will also be reflected at a biochemical level. The effective range of D-cycloserine for the propagation of L-structures seems to be as narrow as for many other antibiotics (Ward, Madoff & Dienes, 1958).

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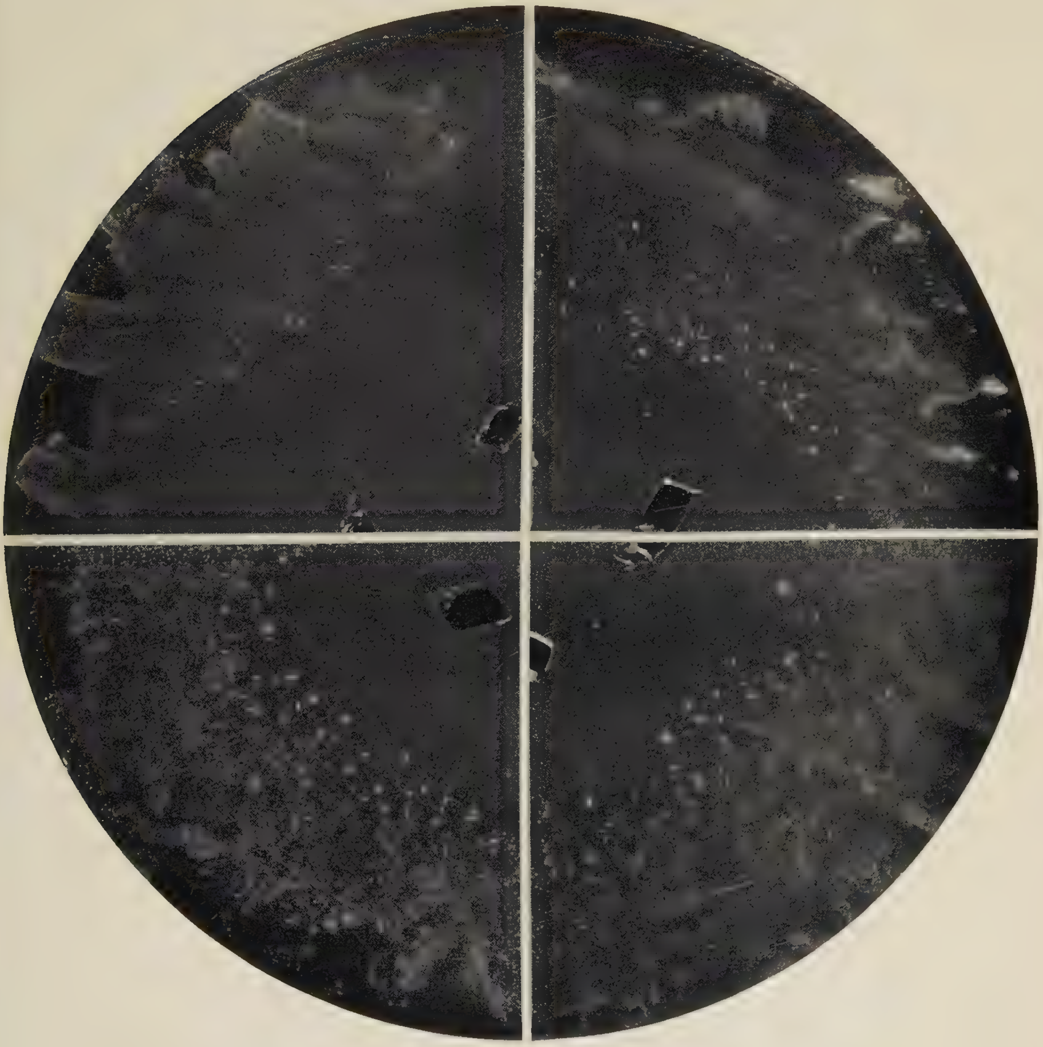
EXPLANATION OF PLATE 1

Quarters of photographs of four plates are assembled to represent the influence of increasing concentrations of D-serine, on the growth of L-phase structures of a group A β-haemolytic streptococcus strain.

The left upper quadrant represents the zone of inhibition obtained with penicillin when the B6 strain was cultured in the basal medium. Some L-phase colonies can be distinguished in the inner zone of bacterial growth.

The next quarters are ordered clockwise. The corresponding plates contained in addition to the basal medium, 0·5, 1, and 1·5 % D-serine respectively. The huge increase of L-phase growth and decrease of bacterial growth in the same strain, caused by the increase of the concentration of the amino acid, is clearly demonstrated.

A peculiarity of L-phase growth of the B6 strain was the diffusion of an unknown brownish substance from the colonies into the surrounding agar. This explains the whitish and rather vague circumference shown by many L-phase colonies of large size.



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Recombination of Characters Between Mutant Stocks of *Vibrio cholerae*, Strain 162

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SUMMARY

Crosses between two mutant strains derived from *Vibrio cholerae*, one requiring valine and isoleucine and the other arginine and purine, yielded recombinants when seeded together on a minimal medium containing L-asparagine. Other markers such as resistance or sensitivity to streptomycin and a lytic choleraphage, which were unselected under the experimental conditions, also segregated in the recombinants.

INTRODUCTION

Previous studies (Bhaskaran, 1958, 1959) have shown that certain strains of *Vibrio cholerae*, when grown together in pairs, gave rise to recombinants which displayed characters derived from both parents. It was also shown that for this occurrence, a fertility factor (designated the *P* factor) should be present in either one or the other parent. This factor was first identified with *V. cholerae*, strain 129. The nature of the fertility factor was obscure but cells of strains possessing it (P^+ strains) produced plaque-like clearings when tested on other strains devoid of this factor (P^- strains). The clearings resembled those produced by bacteriophages but unlike the latter, culture filtrates of P^+ strains did not produce clearings, i.e. contain free phage particles. The possibility that the substance responsible for the clearings produced by P^+ strains may be a bacteriocin has been suggested by Professor J. Lederberg (personal communication).

The P^+ character was infective, i.e. P^- strains, suitably marked to ensure its subsequent recovery from mixed cultures, were rendered P^+ by growth with other P^+ strains. As culture filtrates of P^+ strains were unable to effect this infection it appeared that transfer of the *P* factor occurred between intact cells. Thus the *P* fertility factor in *Vibrio cholerae* was very similar to the fertility factor *F* in the genetic system of *Escherichia coli* (Hayes, 1953*a*; Cavalli, Lederberg & Lederberg, 1953), though F^+ strains of *E. coli* do not produce clearings on F^- strains. Crosses between two P^- strains of *V. cholerae* are infertile as are crosses of two F^- strains of *E. coli*.

The present communication deals with a study of genetic recombination in *Vibrio cholerae* employing mutant stocks derived from strain 162 with complementary nutritional dependencies and differing also in resistance to streptomycin and to a lytic choleraphage (phage 200 Lahiri).

METHODS

Strains. A list of mutants of *Vibrio cholerae*, strain 162, and the technique employed for their isolation is given in Table 1. The symbols used to denote the various mutant characters are those proposed by Dr M. Demerc for 'a uniform nomenclature in bacterial genetics' (see *Microbial Genetics Bulletin*, **16**, 38, 1958). It will be seen that the *ilva* mutant was isolated in a single step which indicates that its nutritional deficiencies, i.e. requirement for isoleucine and valine probably resulted from a single gene mutation.

Table 1. *Mutants of* Vibrio cholerae, *strain* 162

Strain	Genotype	Origin	Technique employed for isolation
162	*pur str-s 200-s*	Parent strain	—
V37	*arg pur str-s 200-s*	162	Ultraviolet and penicillin (Davis, 1949)
V43	*arg pur str-r 200-s*	V37	Selection by streptomycin
V44	*arg pur str-r 200-r*	V43	Selection by phage 200
V45	*arg pur str-s 200-r*	V37	Selection by phage 200
162/p	*pur*$^+$ *str-s 200-s*	162	Selection on minimal medium
V39	*ilva str-s 200-s*	162/p	Ultraviolet and penicillin
V40	*ilva str-r 200-s*	V39	Selection by streptomycin
V41	*ilva str-r 200-r*	V40	Selection by phage 200
V42	*ilva str-s 200-r*	V39	Selection by phage 200

Key. pur: purine requirement (present in wild type strain), satisfied by hypoxanthine; *arg*: arginine requirement; *ilva*: isoleucine + valine requirement; *pur*$^+$: purine not required; *str-s*: streptomycin sensitivity; *str-r*: streptomycin resistance; *200-s*: phage 200 sensitivity; *200-r*: phage 200 resistance.

Media. Nutrient broth and nutrient agar were prepared as described previously (Bhaskaran, 1958).

Recombination techniques. The technique employed in the past was to grow the parent strains together in broth for 18 hr. at 37° and to seed a washed suspension of the mixture on a minimal medium (Bhaskaran & Rowley, 1956) which selected non-exacting 'prototroph' recombinants. Results were negative if the preliminary growth of the mixed strains was omitted, although the period of incubation could be curtailed to 4 hr. without any significant reduction in the number of recombinants recovered. This technique was open to the criticism that the relative frequencies of the various recombinant classes may be influenced by the selective forces in the culture mixture. To obviate this it was necessary to seed washed suspensions of the parent strains directly on to a minimal medium and isolate recombinants produced by recombination *in situ*. As the minimal medium employed was not satisfactory for this purpose it was fortified with L-asparagine (0·004 %, w/v) as Fisher (1957) had shown that a dicarboxylic acid of the Krebs tricarboxylic acid cycle (or an immediate precursor) was essential for the energy required during conjugation and genetic exchange in *Escherichia coli*. This medium proved quite satisfactory and all the experiments recorded in this paper were carried out by direct seeding on minimal media containing L-asparagine.

For these experiments 10 ml. volumes of nutrient broth contained in 100 ml. Erlenmeyer flasks were seeded from overnight agar slant cultures of the strains individually. After 4 hr. incubation at 37° without aeration the cultures were centrifuged at 2500 r.p.m. for 15 min. and the supernatants were discarded. The bacterial deposits were resuspended in 1 ml. of minimal medium and employed in recombination tests. 0·1 ml. amounts of each culture were inoculated separately as controls, while 0·05 ml. amounts of each culture were mixed and spread on the surface of the minimal media for the isolation of recombinants. The plates were incubated at 37° for 72 hr. and the colonies which appeared were counted and investigated further.

Study of recombinants. Colonies which appeared on recombination plates were subjected to three serial cultivations on the same medium as that on which they were isolated. This served to free them from parental contaminants and single colony isolations were not generally carried out. The purified recombinant colonies were then scored for streptomycin sensitivity by spotting on nutrient agar containing dihydrostreptomycin sulphate (500 μg./ml.) and for sensitivity to phage 200 by streaking a phage suspension across a streak of the culture on nutrient agar.

RESULTS

Crosses were carried out between the *ilva* and the *arg pur* stocks of *Vibrio cholerae*, strain 162. Reverse mutations to single nutritional deficiencies were rare in the parent strains (see Table 2) so that every colony which appeared from a cross on a supplemented minimal medium could justifiably be regarded as a recombinant.

Table 2. *Number of colonies on supplemented and unsupplemented minimal agar from* arg pur *and* ilva *mutants of* Vibrio cholerae, *strain* 162, *and from* $P^- \times P^-$ *crosses*

Strain	P polarity	Expt. no.	Medium					
			Minimal		Minimal + purine*		Minimal + arginine*	
arg pur str-s 200-s	P^-	1	Nil	Nil	Nil	1	2	2
		2	Nil	Nil	1	1	4	3
arg pur str-r 200-r	P^-	1	Nil	Nil	Nil	Nil	Nil	Nil
		2	Nil	Nil	Nil	Nil	Nil	Nil
ilva str-s 200-s	P^-	1	Nil	Nil	Not done	Not done	Not done	Not done
		2	Nil	Nil	Not done	Not done	Not done	Not done
ilva str-r 200-r	P^-	1	Nil	Nil	Not done	Not done	Not done	Not done
		2	Nil	Nil	Not done	Not done	Not done	Not done
arg pur str-s 200-s ×	$P^- \times P^-$	1	Nil	Nil	Nil	Nil	1	Nil
ilva str-r 200-r	cross	2	Nil	Nil	Nil	Nil	3	3
arg pur str-r 200-r ×	$P^- \times P^-$	1	Nil	Nil	Nil	1	Nil	Nil
ilva str-s 200-s	cross	2	Nil	Nil	Nil	Nil	Nil	Nil

* Purine (hypoxanthine) and arginine were added to give a final concentration of 0·05 % (w/v). 5×10^8 cells of each parent strain were seeded together on each plate in crosses and 10^9 cells of each strain individually in other tests.

Figures represent colony counts on duplicate plates.

Details of experiments carried out to show the infertility of $P^- \times P^-$ crosses are also given in Table 2. As the strains were initially P^- it was possible to carry out crosses after rendering either parent P^+. Crosses were also carried out with the *str*, *200* markers reversed in the parent strains. Thus four crosses were possible; for details see Table 3.

Table 3. *Numbers of recombinant colonies from crosses between* arg, pur *and* ilva *mutants of* Vibrio cholerae, *strain* 162, *on supplemented and unsupplemented minimal agar*

Medium	Parents	*arg pur str-r 200-r* × *ilva str-s 200-s*		*arg pur str-s 200-s* × *ilva str-r 200-r*	
	Cross Number	1	2	3	4
	P polarity	$P^+ \times P^-$	$P^- \times P^+$	$P^+ \times P^-$	$P^- \times P^+$
	Expt. no.				
Minimal	1	20, 25	1, 2	18, 13	Nil, Nil
	2	44, 33	14, 4	21, 17	Nil, Nil
Minimal + purine*	1	207, 176 *pur* 44 %	166, 200 *pur* 86 %	228, 280 *pur* 92 %	258, 262 *pur* 100 %
	2	320, 270 *pur* 22 %	286, 332 *pur* 98 %	88, 100 *pur* 88 %	310, 270 *pur* 100 %
	3	186, 154 *pur* 43 %	Not done	232, 244 *pur* 92 %	Not done
Minimal + arginine*	1	15, 11 *arg* nil	57, 117 *arg* 100 %	32, 80 *arg* 78 %	51, 44 *arg* 98 %
	2	8, 9 *arg* 24 %	48, 36 *arg* 94 %	47, 52 *arg* 42 %	27, 67 *arg* 100 %
	3	22, 17 *arg* 3 %	Not done	22, 36 *arg* 34 %	Not done
Minimal + valine*	1	40, 32 *val* nil	Nil, Nil	13, 26 *val* nil	Nil, Nil
	2	36, 55 *val* nil	1, nil *val* nil	19, 23 *val* nil	Nil, Nil

5×10^8 cells of each parent strain were seeded together on each plate: the figures represent colony counts on duplicate plates. The percentages of *pur* and *arg* colonies were calculated after testing 50 to 100 colonies from each cross on the supplemented media. When the total number of colonies was less than 50, all the colonies were tested.

* Purine (hypoxanthine), arginine and valine were added to give a final concentration of 0·05 % (w/v).

In each experiment recombinants were isolated on minimal medium and also on minimal medium supplemented with a single nutritional factor, i.e. purine, arginine, valine or isoleucine. This study would show the relative frequency of the various recombination classes in respect of the unselected markers *str*, *200* and *arg* or *pur* under the same conditions of test.

It will be seen from Table 3 that no *val* recombinants were isolated from any of the four crosses, as all colonies which appeared on valine supplemented media were prototrophs. Tests with isoleucine media (not included in the table) also yielded only prototrophic recombinants. This was not unexpected as it was obvious by the very isolation of the *ilva* mutant that its two nutritional deficiencies were governed by the same locus.

The occurrence of prototrophic recombinants was significantly influenced by P polarity. *ilva* P^+ × *arg pur* P^- crosses (crosses 2 and 4, Table 3) yielded few prototrophs when compared to the same cross reversed with respect to P (crosses 1 and 3, Table 3). *pur* recombinants were significantly more numerous on media supplemented with purine than were prototrophs on the same medium or on unsupplemented minimal media. (Cross 1 was to some extent an exception to this as prototrophs were more frequent than *pur* recombinants. *arg* recombinants were also few in this cross.) This indicated that *pur* and *arg* segregated independently.

Table 4. *Segregation of* str *and* 200 *in recombinants arising from various parental combinations*

	Parents ...	*arg pur str-r 200-r* × *ilva str-s 200-s*		*arg pur str-s 200-s* × *ilva str-r 200-r*	
	Cross number	1	2	3	4
Recombinant class	P polarity	$P^+ \times P^-$	$P^- \times P^+$	$P^+ \times P^-$	$P^- \times P^+$
pur$^+$ *arg*$^+$ *ilva*$^+$	Expt. no.				
	1	142 *str-s 200-s*	10 *str-s 200-s*	74 *str-r 200-r* 1 *str-s 200-s* 1 *str-s 200-r*	—
	2	152 *str-s 200-s* 1 *str-r 200-s*	16 *str-s 200-s* 4 *str-r 200-r* 1 *str-r 200-s*	114 *str-r 200-r* 1 *str-s 200-s* 1 *str-r 200-s* 1 *str-s 200-r*	—
	3	94 *str-s 200-s*	Not done	34 *str-r 200-r* 2 *str-r 200-s* 1 *str-s 200-r*	Not done
pur arg$^+$ *ilva*$^+$	1	22 *str-s 200-s*	36 *str-r 200-r* 5 *str-s 200-s* 2 *str-r 200-s*	43 *str-s 200-s* 2 *str-r 200-r* —	47 *str-s 200-s* — —
	2	10 *str-s 200-s*	49 *str-r 200-r*	55 *str-s 200-s* 12 *str-r 200-r* 1 *str-s 200-r* 2 *str-r 200-s*	49 *str-s 200-s* 1 *str-r 200-s* — —
	3	40 *str-s 200-s* 3 *str-r 200-s*	Not done	42 *str-s 200-s* 3 *str-r 200-r* 1 *str-r 200-s*	Not done
arg pur$^+$ *ilva*$^+$	1	—	49 *str-r 200-r* 1 *str-s 200-r*	37 *str-s 200-s* 1 *str-r 200-s*	45 *str-s 200-s* 1 *str-r 200-r* 2 *str-r 200-s*
	2	—	35 *str-r 200-r*	30 *str-s 200-s* 1 *str-r 200-r*	49 *str-s 200-s* 1 *str-r 200-s*
	3	—	Not done	17 *str-s 200-s*	Not done

Figures represent the number of colonies examined.

Table 4 presents an analysis of recombinants, isolated from crosses recorded in Table 3, with respect to the unselected markers *str* and *200*. It will be seen that the vast majority of prototrophs (636 out of 650 colonies) from all the crosses yielding prototrophs, i.e. crosses, 1, 2 and 3, had the *str*, *200* markers of the *ilva* parent irrespective of its P polarity. This suggests that these characters segregated together with the *arg*$^+$ and *pur*$^+$ markers of the *ilva* parent and probably occupied a place between them in the chromosome.

The segregation of *str*, *200* markers in *pur* and *arg* recombinants was peculiar. In crosses 2 and 4 (Table 4) which are alike as regards P polarity of the parent strains and differ only in the *str*, *200* markers of the parents, *arg* and *pur* recombinants generally resembled the *arg pur* parent as regards *str*, *200* markers. When crosses 1 and 3 (Table 4) are similarly compared it will be seen that in cross 1, the *pur* recombinants generally resembled the *ilva* parent in *str*, *200* markers, while in cross 3 the resemblance was with the *arg pur* parent. As already observed, *arg* recombinants were few in cross 1 and could not be examined in sufficient numbers. *arg* recombinants of cross 3 were, however, similar to the *pur* recombinants of the same cross in possessing the *str*, *200* markers of the *arg pur* parent.

This difference in the segregation of *str*, *200* markers between crosses 1 and 3 (Table 4) could not be explained and further experiments were carried out to study recombinants obtained from crosses between the parent strains in which *str*, *200* markers were introduced in different combinations (Table 5).

Table 5. *Segregation of* str *and* 200 *in other crosses*

Cross no.	Parents	Expt. no.	Number of colonies on minimal agar + purine	Recombinants	
				arg^{+} *pur*$^{+}$ *ilva*$^{+}$	*pur arg*$^{+}$ *ilva*$^{+}$
1	*arg pur str-s 200-r* P^{+} × *ilva str-r 200-s* P^{-}	1	180, 182	49 *str-r 200-s*	46 *str-r 200-s*
			pur 50 %	—	4 *str-s 200-r*
		2	166, 200	23 *str-r 200-s*	19 *str-r 200-s*
			pur 47 %	—	9 *str-s 200-r*
				—	1 *str-s 200-s*
2	*arg pur str-r 200-s* P^{+} × *ilva str-s 200-r* P^{-}	1	188, 222	11 *str-s 200-r*	24 *str-r 200-s*
			pur 72 %	3 *str-s 200-s*	12 *str-s 200-r*
		2	202, 252	21 *str-s 200-r*	42 *str-r 200-s*
			pur 69 %	2 *str-s 200-s*	12 *str-s 200-r*
				2 *str-r 200-s*	1 *str-r 200-r*

Though the number of colonies examined in these experiments (Table 5) was fewer than in previous crosses it is significant that when the *200-r* marker was introduced in the *arg pur* P^{+} parent, 82 % of *pur* recombinants had the unselected *str*, *200* markers of the *ilva* parent (like cross 1, Table 4), while if the *arg pur*, P^{+} parent was *200-s*, only 26 % of *pur* recombinants were like the *ilva* parent in the *str*, *200* markers (like cross 3, Table 4). This interesting observation requires further study to determine if the aberration in segregation pattern was consistently associated with phage 200 resistance in the *arg pur* P^{+} parent.

A study of Tables 4 and 5 also reveals the fact that the *str* and *200* markers generally segregate together in the various recombinant classes (1595 colonies out of 1625 tested). Only 30 colonies show cross-overs between *str* and *200*. This strongly suggests close linkage of the *str*, *200* loci in *Vibrio cholerae*, strain 162.

DISCUSSION

The occurrence of genetic recombination between two auxotrophic mutants of *Vibrio cholerae*, strain 162, is demonstrated by the isolation of recombinants employing selective minimal media in which neither parent strain will grow. The role of a fertility factor in this genetic system, the *P* factor (Bhaskaran, 1959), is emphasized and genetic exchange appears to result from cellular conjugation between parental cells. The simultaneous segregation of certain other unselected markers like resistance or sensitivity to streptomycin and phage 200 also renders this phenomenon different from DNA induced transformations (pneumococcus, Avery, McLeod & McCarty, 1944; *Haemophilus influenzae*, Alexander & Leidy, 1951) and phage-mediated transductions in Salmonella (Zinder & Lederberg, 1952) in which only isolated characters are usually transferred.

As the strains used in crosses were all derived by distinct mutational steps from a common ancestor the marked characters are assumed to be allelic, though actual tests for allelism have not been carried out. The recombination data suggests close linkage of the loci controlling resistance to streptomycin and phage 200.

The role of the fertility factor *P* in this genetic system is as obscure as *F* in *Escherichia coli*. As in *E. coli*, transfer of the *P* factor in *Vibrio cholerae* is independent of the other genetic markers and occurs far more frequently. All the recombinants examined so far are P^+ as shown by their associated lytic effects on P^- strains as well as by their fertility in crosses with P^- strains. No P^- recombinants have so far been isolated.

In *Escherichia coli*, F^+ strains are regarded as gene donors contributing only segments of their chromosomes to F^- strains which are regarded as gene acceptors (Hayes, 1953*b*). An alternative explanation has also been put forward which explains the aberrations in segregation as due to segmental elimination in the zygotes which occurs preferentially from the F^+ chromosome (Lederberg, 1955). The evidence so far available in the genetic system of *Vibrio cholerae* does not permit any speculation on this point and work is in progress to study the mechanism of this recombination.

It was reported earlier (Bhaskaran, 1959) that in certain inter-strain crosses in *Vibrio cholerae*, some prototrophic colonies obtained were unstable and consistently segregated a parental (auxotrophic) type and recombinants. Such unstable prototrophs which appeared to be made up, at least in part, of partial heterozygotes (heterogenotes) could be identified by their agglutinability with both Inaba and Ogawa ‘O’ antisera when the parent strains differed in antigenic type (either Inaba or Ogawa). In the present investigation only Ogawa type cultures were employed in crosses and the identification of persistent heterogenotes by agglutination tests was not possible. It was not feasible to examine all prototrophs obtained from crosses to see if any of them segregated parental auxotroph types. It is quite likely that such colonies, if present, might have been missed in this study.

Further work is indicated to ascertain the true nature of the *P* factor in *Vibrio cholerae*. However, it has been observed that this factor is rather unstable. From P^+ cultures maintained on nutrient agar slants for over three months it has consistently been possible to isolate P^- variants. For the satisfactory maintenance of P^+ strains it is desirable to stock the cultures at 4° in a refrigerator and to make weekly transfers to fresh media.

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Denitrification as Influenced by Photosynthetic Oxygen Production

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SUMMARY

An attempt was made to detect interaction between photosynthesizing algae and denitrifying bacteria by cultivating *Chlorella* sp. with *Pseudomonas stutzeri*. Denitrification was inhibited by illumination of the mixed culture after a dark period of 3 hr. It is suggested that immediate inhibition is due to the molecular oxygen produced by the suspended algae and distributed homogeneously in the culture. A delay of this inhibition may be caused by uptake of oxygen by the denitrifiers at a more rapid rate than its production by the algae. Thus, the quantitative aspects of this interaction depend upon the particular conditions of cultivation used. The ecological implications are discussed.

INTRODUCTION

In shallow highly productive lakes and estuaries the daily gaseous exchanges due to photosynthesis can often produce a zone of reducing conditions above the bottom. Such waters are characterized by the occasional elimination of oxygen deficiencies by the photosynthetic action of submerged plants. Consequently, a direct inhibition of anaerobic processes may occur when photosynthetic liberation of oxygen is possible in the same medium under the same conditions of growth.

A situation of this type was tested *in vitro* by cultivation of algae with a denitrifying bacterium which was found as a contaminant of algal cultures. The work was stimulated by the observation of vigorous gas production in liquid cultures of a *Chlorella* sp. still contaminated by bacteria, which had been accidentally stored in the dark for several days. The gas was found to consist of CO_2, 10–15% (v/v) N_2, and traces of O_2 and N_2O. Nitrate, initially present in the culture at 0·3% (w/v), disappeared in a period of about 14 days. The only hydrogen donor present and possibly used by the denitrifying micro-organisms must have been decomposing algal material. Addition of other hydrogen donors (e.g. peptone, yeast extract) vigorously accelerated the disappearance of nitrate. Shaking and aeration retarded, but did not prevent, the loss of nitrate and appearance of elementary nitrogen. The sensitivity of the denitrification to an accompanying algal photosynthesis was then studied.

Since the discovery of denitrification, its occurrence in the presence of oxygen has been discussed in many papers which often reported contradictory results.

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Failure to detect an inhibition effect of molecular oxygen on denitrification was found to be due to the method of estimating the dissolved oxygen in liquid media on the basis of a supposed equilibrium with the gas phase. Having applied polarographic measurements of the dissolved oxygen during denitrification Skerman & MacRae (1957*a*) disproved the existence of such an equilibrium in cultures containing heavy suspensions of organisms and—implying the alternative use of molecular oxygen and nitrate as hydrogen acceptors for denitrifying bacteria—they concluded: 'It appears that the competition between oxygen and nitrate for the donor electrons so favors the oxygen that nitrate reduction only occurs when the supply of oxygen is inadequate to meet the demand.' In a study on denitrification and micro-zonal oxygen deficiencies in an aerated medium (Jannasch, 1960), denitrification was determined by measuring the molecular nitrogen and nitrous oxide liberated into the gas phase. According to the findings of Skerman, Lack & Millis (1951) and Skerman & MacRae (1957*a*, *b*), the formation of gaseous nitrogen compounds may serve as the indicator for the availability of molecular oxygen to denitrifying bacteria. By this means the influence of oxygen produced by photosynthesizing algae upon denitrification was studied and is described in the present paper.

METHODS

Organisms. A bacteria-free culture of *Chlorella* sp. was obtained by streaking a concentrated sample of water from Lake Mendota, Madison, Wisconsin, U.S.A., on mineral agar containing soil extract (Pringsheim, 1946), and by several subsequent transfers on this medium. A loopful from an active agar slope culture thus obtained was inoculated into 500 ml. liquid medium (Umbreit, Burris & Stauffer, 1957); diluted tenfold, and the culture was aerated in wash bottles (5% (v/v) CO_2; Warburg, Burk, Schocken & Hendricks, 1950) at 20°; light of about 1200 lux was supplied by fluorescent tubes. After 3 days the organisms in the culture were removed by centrifugation, washed, and suspended in 500 ml. fresh medium. The nitrate concentration in the medium was adjusted to 200 mg./l., and 800 mg. yeast extract/l. was added. The initial concentration of algae was *c.* 200,000/ml.

Pseudomonas stutzeri was isolated and purified by the method of van Niel & Allen (1952) from algal cultures stored for several days in the dark (see above). Half of the bacteria used for inoculum were grown aerobically, the other half anaerobically, for 12 hr. at 20° and subsequently filtered, washed, and suspended in a few ml. phosphate buffer (pH 7·0). The initial density of viable bacteria in the culture was about 10^6/ml.

Cultivation. The cultivation of *Chlorella* sp. with *Pseudomonas stutzeri* was accomplished in a flat culture vessel connected with a gas-collecting burette and levelling bulb (Fig. 1, *C*, *G*). After inoculating the algal culture with the bacteria, helium containing 5% (v/v) CO_2 was bubbled through the liquid; a sterile cotton filter was used to sterilize the gas entering through tube *A*. Most of the oxygen was swept out of the system by a continuous gas stream supplied for 20 min. Then the volume of the gas phase was adjusted to 500 ml., and the system closed. The entire culture system was placed in a 20° incubator in which it was illuminated as described. Since gaseous oxygen was not present in concentrations great enough to influence denitrification, circulation of the gas phase was not necessary. Gentle agitation of the culture with a magnetic stirrer (Fig. 1, *M*) was used to prevent sedimentation. Growth took place at somewhat reduced pressure (Fig. 1, *R*). The change of the gas volume was measured after adjustment to 760 mm. mercury (Nilsson, 1941). Gas samples were taken after evacuation of tube *T* and sampling bottle *S*, and were analysed with the mass spectrometer. After absorption of CO_2, increase or decrease in volume of single gas components (N_2, O_2, N_2O) was calculated from the change of their proportions in the gas mixture, and from the change of the entire gas volume. Tests for hydrogen were made according to Milton & Waters (1956). Nitric oxide was found by the method of Najjar & Allen (1954) to be present in insignificant amounts and was subsequently neglected. When complete gas absorption was necessary, as for the detection of hydrogen and nitric oxide, a circulating system was used as illustrated by Jannasch (1960). Liquid samples for the determination of nitrate and nitrite were taken through tube *A* with the aid of the levelling bulb; care was taken to avoid contamination of the culture.

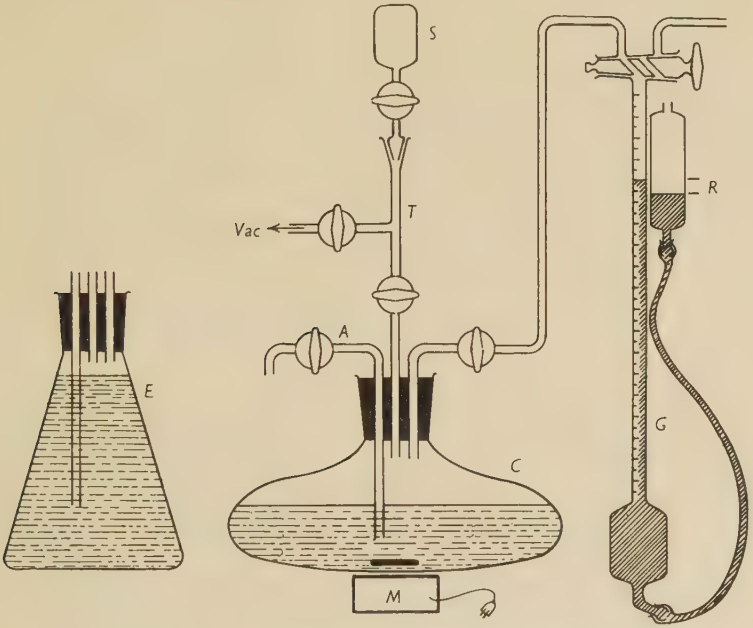

Fig. 1. Apparatus used (for explanation see text).

RESULTS

When the freshly inoculated medium was exposed to light after a 3 hr. dark period, oxygen was found to be liberated into the gas phase (Expt. 1; Fig. 2). The formation of elementary nitrogen, however, did not cease until about 20 min. after the oxygen concentration had reached an almost constant value of 0·48 % (v/v). Subsequently, the N_2-concentration did not change during more than 2 hr. Then a slow and irregular increase in N_2 was found, while the O_2-concentration did not change significantly. Nitrous oxide (see Fig. 5) was here calculated as N_2. The results of four parallel experiments showed a fair agreement, except for the last period of N_2-formation.

An attempt was made to simplify this experiment by splitting it into four modified experiments:

Expt. 2*a*. *Chlorella* inoculated only; conditions of cultivation unchanged.

Expt. 2*b*. *Pseudomonas stutzeri* inoculated only; conditions of cultivation unchanged.

Expt. 2*c*. Both organisms inoculated; no light but 20% (v/v) O_2 supplied after 3 hr.

Expt. 2*d*. Both organisms inoculated; 20% (v/v) O_2 supplied at the beginning of the experiment.

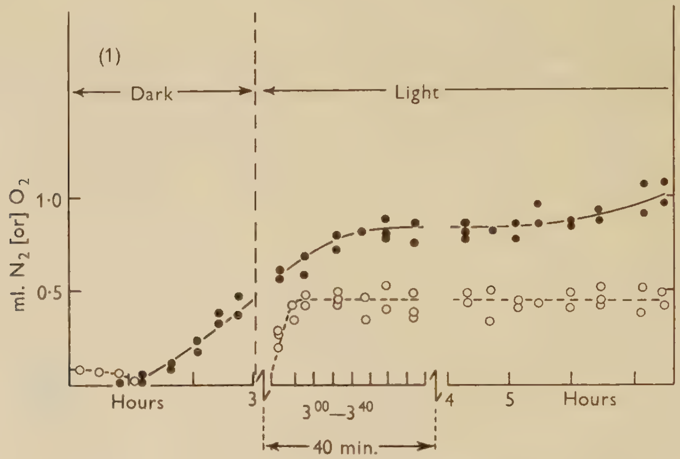

Fig. 2. Expt. 1: ●, N_2; ○, O_2 (for explanation see text).

The results were as follows:

Expt. 2*a*. As in Expt. 1 0·14% (v/v) O_2 was found in the gas phase at the beginning of the experiment; it decreased to about 0·08% (v/v) during the dark period. When the first sample within the light period was taken 1 min. after illumination, the O_2-concentration was as high as 1·8% (v/v) and subsequently increased (Fig. 3, Expt. 2*a*).

Expt. 2*b*. The O_2-concentration decreased to zero within about 90 min. Starting after 40 min., N_2-formation was unaffected by illumination, and did not cease until all nitrate was used up after 27 hr. (Fig. 3, Expt. 2*b*).

Expt. 2*c*. The N_2-formation was completely stopped a few minutes after addition of 20% (v/v) of O_2 to the gas phase. Without any apparent lag, the O_2-concentration decreased. The N_2-formation continued when the O_2-concentration was as low as 7·1% (v/v) (Fig. 4, Expt. 2*c*).

Expt. 2*d*. The O_2-concentration decreased from the beginning. After illumination, the decrease continued, but at a slower rate. N_2-formation did not begin before an O_2-concentration of 4·2% (v/v) was reached after about 11 hr. As compared with Expts. 2*b* and 2*c*, the N_2-formation appeared to be depressed.

A few further observations may be noted. As far as the sensitivity of this volumetric method allowed, in Expt. 2*b* the formation of 1 mmole N_2 from 2 mmole nitrate was found, as described by Allen & van Niel (1952). Calculating on the same basis, there was a small 'loss' of nitrate in Expts. 1, 2*c* and 2*d*. After 10 hr., this probable nitrate reduction by the algae did not exceed 4 mg. NO_3^-/l. or 2% (w/v) of the nitrate initially present. Ammonia and amino-nitrogen, present in the yeast extract, appeared to be a sufficient nitrogen source for *Pseudomonas stutzeri*, and seemed to provide nitrogen for the algae as well.

Nitrous oxide was found up to 6·5% (v/v) of the N_2-concentration in the gas phase. While in Expt. 1 nitrite increased almost constantly up to 2·9 mg./l. (Fig. 5), nitrous oxide showed a distinct decrease between 5 and 8 hr. This is a correction of a preliminary non-reproducible observation (Jannasch, 1959) in which nitrous oxide was found to be formed during the dark period.

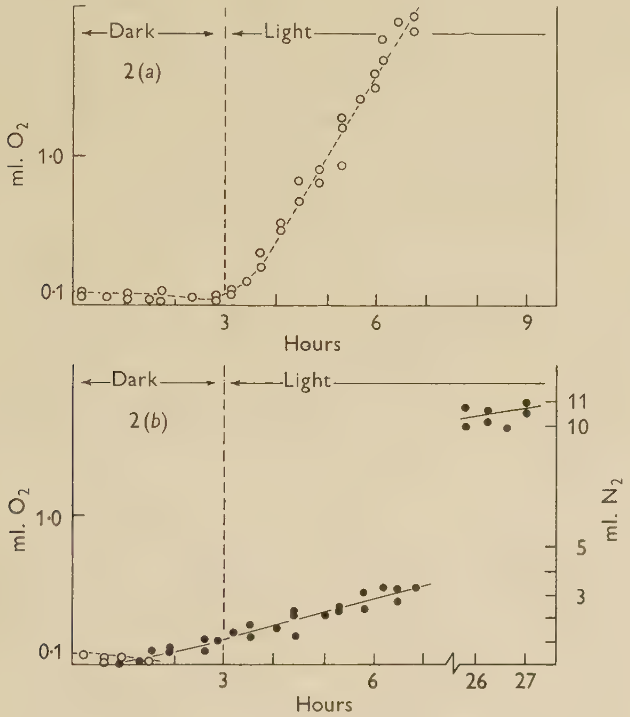

Fig. 3. Expts. 2*a*, *b*: ●, N_2; ○, O_2 (for explanation see text).

The possible formation of elementary nitrogen from the reaction between nitrite and nitrogenous compounds reduced below the N_2-level, as reported occasionally, was disproved for the conditions of cultivation used in the present study. After the addition of several ^{15}N-labelled α-amino acids, no $^{15}N_2$ was found in the gas phase.

After a week of cultivation the count of the algae in the simultaneous culture with *Pseudomonas stutzeri* was 20–30% higher than that of the pure culture of algae, and increased to 80–100% higher after 19 days.

DISCUSSION

Since no oxygen was found in the gas phase during the second and third hour of the dark period, and from consideration of Henry's law of partial pressures and the relative sensitivity of the mass-spectrometer test, the culture seemed to be deprived of dissolved oxygen. There is, however, no direct evidence to support this conclusion. Rabinowitch (1956), in reviewing the literature up to 1956, did not report any method for depriving an algal culture of oxygen other than by prolonged sweeping with oxygen-free gas in an open system. Since in the present experiments the first sample was taken 1 min. after illumination, no induction period shorter

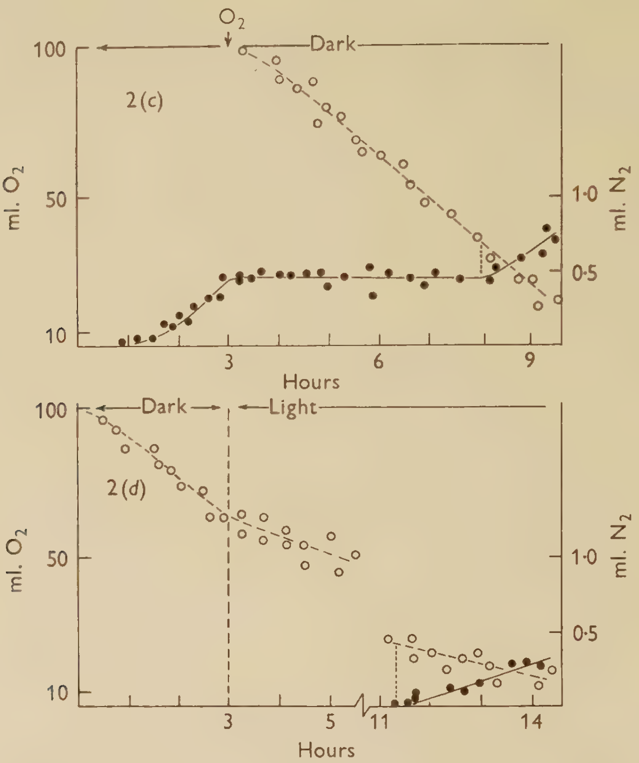

Fig. 4. Expts. 2*c*, *d*: ●, N_2; ○, O_2 (for explanation see text).

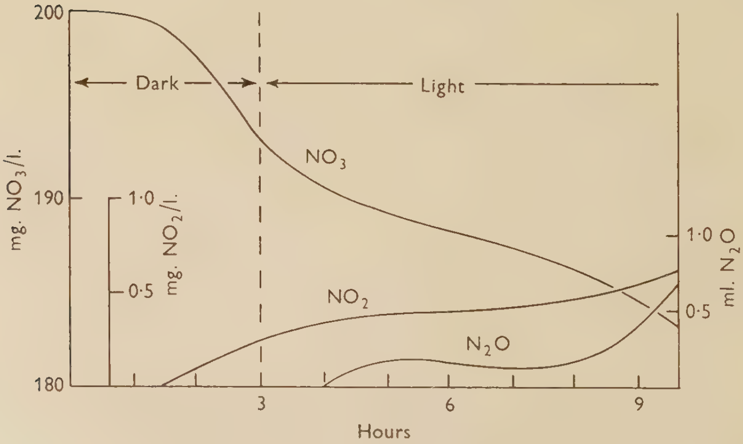

Fig. 5. Transformation of inorganic nitrogen in Expt. 1.

than 1 min. could be determined. The amount of oxygen liberated at that time appeared to be slightly higher in Expt. 2*a* than in Expt. 1.

Two related applications of physiological tests may be considered. Oxygen production by green algae was observed in the classical experiments of Engelmann (1881), who used semi-anaerobic conditions with motile O_2-requiring bacteria as an indicator. Harvey (1928), using luminous bacteria, found that oxygen was produced by algae under completely anaerobic conditions within a second after the beginning of illumination. The similarities of photosynthesis in green plants and in purple bacteria led to the notion that oxygen is not necessary for this process; this aspect was presented by Franck (1953), and by Arnon, Whatley & Allen (1957).

On the other hand, inhibition of oxygen production following anaerobic incubation in the dark, has frequently been observed. It was claimed by Franck, Pringsheim & Lad (1945), and by Pratt, Oneto & Pratt (1945) that such inhibition was caused by changes in pH value due to the accumulation of products of fermentation. With Chlorella Noack, Pirson & Michels (1939) showed that neutralization as well as aeration removed the anaerobic inhibition; the oxidizable substance was unknown. Within periods of cultivation used in the present experiments, Chlorella has not been found to be adaptable to photo-reduction. No hydrogen was liberated as tested in Expt. 1.

The experiments showed clearly that the oxygen produced by the algae inhibited the formation of N_2 from nitrate by *Pseudomonas stutzeri*. The quantitative aspects of this interaction, however, are believed to be influenced by the particular cultural conditions. The relative high concentration of readily oxidizable organic matter is responsible for the fact that the O_2-uptake by the bacteria exceeds considerably the O_2-production by the algae. Within the first 20 min. of the light period, oxygen was produced by the algae and subsequently used as a hydrogen acceptor along with nitrate. After 20–30 min., oxygen seemed to replace nitrate until denitrification stops completely. Whether this delay of the complete inhibition of N_2-formation was due to a slow increase of O_2-production is not apparent. The appearance of oxygen in the gas phase does not reflect the true rate of O_2-production but only that small fraction which 'escapes' into the gas phase before being consumed by the bacteria. The fact that a constant O_2-concentration in the gas phase is reached in Expt. 1 shortly after illumination, supports the assumption that an equilibrium has been formed between liberation and re-absorption of oxygen by the liquid phase.

The beginning of the N_2-formation occurred in the presence of quite different O_2-concentrations, depending apparently upon whether oxygen was supplied in the gas phase or produced by the suspended algae. According to Skerman's conclusions (see introduction) this is to be expected if the algae actually provide a more or less homogeneous distribution of the oxygen produced. Measuring the dissolved oxygen under the most favourable conditions for its uniform distribution, Skerman *et al.* (1957*b*) found 0·2 mg. O_2/l. sufficient to inhibit denitrification completely. When, however, oxygen was determined in the gas phase (Sacks & Barker, 1949), only a partial inhibition of denitrification was found at O_2-concentrations as high as 4–5 % (v/v, *c.* 2·0 mg./l.). This implies the existence of a gradient in oxygen concentration from the gas phase to the liquid phase, depending upon the rate of oxygen uptake in the latter. When oxygen is supplied directly into the liquid phase, as in the case of suspended algae under illumination, a lower oxygen-percentage in the gas phase must appear as the limiting concentration for denitrification.

The role of inadequate oxygen supply in determining the inhibitory effect of molecular oxygen on denitrification was studied by Collins (1956) and Fedorov & Sergeeva (1957). Following the recommendations of these workers, a more vigorous agitation and culture vessels of different shape (providing a smaller liquid surface; Fig. 1, *E*, present paper) were used in repeating Expt. 1. No marked influence on the rate of inhibition was then found. This was not surprising since the oxygen, liberated into the gas phase, was below the inhibitory concentration. These results show that the proposed interaction between photosynthesizing algae and denitrifying bacteria exists in simultaneous culture. In the natural habitat, several additional factors may operate to change the quantitative aspects.

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The Utilization by Yeasts of Acids of the Tricarboxylic Acid Cycle

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SUMMARY

Growth and respiration on intermediates of the tricarboxylic acid cycle as sole sources of carbon were studied with a number of different yeasts. The incorporation of [1:2-^{14}C]acetate by acetate-respiring cells was studied with yeasts capable of utilizing exogenously supplied cycle intermediates (K+) and also with those unable to do so (K−). Enzyme systems were examined by using cell-free extracts. It was concluded that the difference between K+ and K− yeasts was not one of a major metabolic pathway, but probably of the permeability of the intact cells. Evidence was found for the operation of the tricarboxylic acid cycle in yeasts under differing growth conditions, and for the operation of the glyoxylate cycle in yeasts growing on acetate.

INTRODUCTION

Various workers have tested the ability of yeasts to utilize acids of the tricarboxylic acid (TCA) cycle. For example, Leberle & Will (1910) indicated that four strains of *Candida mycoderma* were probably capable of utilizing succinic and malic acids for growth. Okunuki (1931) reported that species of the genus *Rhodotorula* and the genus *Sporobolomyces* seemed able to use citric, succinic and malic acids. In these experiments peptone was present in the growth medium in addition to the organic acid being tested. In 1948, Wickerham & Burton reported that many yeasts could grow with intermediates of the TCA cycle as sole sources of carbon, utilizing citric, succinic, fumaric and malic acids. Wickerham (1951) described 15 species of the genus *Hansenula*; strains of 12 of these species were able to grow on citric or succinic acids. Testing with citrate, succinate and malate, Kudriavzev (1954) found that 36 out of 68 species of ascosporogenous yeasts utilized at least succinic acid. Such intermediates of the TCA cycle are now used in routine identification by the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS). Yeasts may thus be divided into two groups: 'K+', those capable of using as sole carbon source for growth or respiration one or more intermediates of the TCA cycle; 'K−', those yeasts unable to do so.

METHODS

Media. Yeasts were grown in the carbon-free defined medium (A_2) of Barnett & Ingram (1955), to which was added respectively, glucose (A_2G), succinate (A_2S), acetate (A_2Ac) or citrate ($A_2Cit.$) to final concentrations of 1 % (w/v). In addition, Difco Bacto Y–M broth and agar were used where indicated.

Organisms. Stock cultures were maintained at +1° on slopes of Y–M agar and subcultured every 6 months (cf. Kirsop, 1954). The yeasts were incubated at 25°.

The yeasts came from the following collections: The Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Holland (CBS); The National Collection of Yeast Cultures, Great Britain (NCYC); The American Type Culture Collection (ATCC); The North Regional Research Laboratories, Peoria, Illinois, U.S.A. (NRRL); The Carlsberg Laboratory, Copenhagen, Denmark (CARL); The Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada (DBDR); The Low Temperature Station, Cambridge, England (LTS).

The strain of *Saccharomyces drosophilarum*, CBS 2896, was received from Professor V. I. Kudriavzev (Institute of Microbiology, Academy of Sciences of the U.S.S.R.) as *Zygofabospora krasilnikovi* (see Kudriavzev, 1954) and deposited with the CBS.

Growth of yeasts for inocula and suspensions

For growth experiments. For each growth experiment, a fresh Y-M agar slope was inoculated from the stock culture. After incubation for 2 days, the yeasts were washed off the slope with medium A_2 and the entire crop used to inoculate a Roux bottle containing about 100 ml. of A_2G agar. The cultures were harvested after incubation for 2 days.

For manometric experiments. For each manometric experiment a 2-day culture from a Y-M agar slope was used to inoculate a 1 l. Fernbach culture flask, containing 200 ml. medium. These flasks were shaken (at *c.* 2 cycles/sec.; 5 cm. travel) for *c.* 24 hr. at 25°.

For isotope experiments. The suspension of yeast cells was in each case prepared exactly as for manometry; but four Fernbach flask cultures were necessary for each experiment.

For preparation of cell-free extracts. The several grammes of yeast needed in each experiment were grown in a Hoover washing machine adapted for this purpose. The machine consisted of a stainless steel vessel (30 l. capacity) with a mechanical stirrer, and a hole in the bottom of the tank through which sterile air was pumped. A metal top and gasket prevented contamination from the air; heat produced metabolically (Winzler & Baumberger, 1938) was prevented from increasing the temperature of the medium above that of the thermostatically controlled room by means of a cooling coil. The yeast was grown for 24 hr. in media A_2Ac, A_2Cit, or A_2S. The crop was harvested with a Sharples Laboratory Super-centrifuge, washed in 10 l. of 0·9 % (w/v) potassium chloride, re-centrifuged and stored at −15°.

Growth experiments

The methods used were based on those described by Barnett & Ingram (1955).

Auxanography. Each culture was harvested into 10 ml. medium A_2 and transferred to a '1 oz.' (25 ml.) screw-capped bottle by means of a sterile pipette. The concentration of cells was checked roughly with a nephelometer and adjusted aseptically to *c.* 5×10^8 cells/ml. Of this suspension, 0·5 ml. was added to a tube containing 10 ml. melted A_2 agar medium which had been cooled to 48°. The contents of the tube were poured into a Petri dish (9 cm. diameter) after quick but thorough mixing. The surface of the agar was dried by incubating the dishes, open and inverted, at 37° for 1·5 hr. The carbon sources were placed on the surface with a platinum spoon in 2–5 mg. quantities, not more than three test-substances/plate. The auxanograms were incubated and examined every 2 days for a week.

T-*tubes*. The inocula were prepared exactly as for auxanograms. The suspension of cells was added to 400 ml. A_2 medium contained in a sterile 500 ml. Quickfit interchangeable round flask with a flat bottom and short neck, stoppered with a gauze-covered cotton wool plug. This was then attached to a sterile automatic burette, a modification of the inoculating apparatus described by Barnes (1950), for distributing 10 ml. inoculated medium into each T-tube. When only a few tests were to be made on any one strain of yeast, smaller quantities were prepared and delivered with 10 ml. sterile pipettes.

The carbon-sources were distributed with a sterile pipette to each T-tube to a final concentration of *c.* 1 %. They were made up and stored as 10 % (w/v) solutions at pH 5·0 and sterilized through a membrane filter. (This apparatus was Model Stefi 4 of Membranfilter Gesellschaft, Göttingen, Germany, and the bacteria-proof membrane their Spezial-Membranfilter Co 5. For distributing the sterile solutions, the filter was set up as recommended for a Seitz filter by Mackie & McCartney, 1956).

The T-tubes were shaken (*c.* 2 cyc./sec., 5 cm. travel) at 25°, readings being taken with an E.E.L. nephelometer at 0, 2, 4 and 6 days. The nephelometer used was set to give a reading of 52 on the scale with the solid standard provided.

Manometric experiments

For measurements of the oxidation of acetate and of intermediates of the TCA cycle, the centrifuged yeast was suspended in 0·1 M-potassium phosphate buffer (pH 5·9) and re-centrifuged. The washed yeast was re-suspended in 0·1 M-phosphate buffer, pH 5·9, to a final concentration equivalent to *c.* 5 mg. dry wt./ml. This suspension was placed in a Fernbach flask and shaken again at 25° for 1 hr. in order to decrease the rate of endogenous respiration. The rates of oxygen uptake on addition of substrates were measured with Warburg manometers. Flasks (approx. vol. 20 ml.) with single side arms contained 2 ml. yeast suspension in the main compartment, and 25 μmole of the substrates in 0·1 ml. water in the side arms. Strips of Whatman no. 40 filter paper and 0·2 ml. of 2 N-KOH were placed in the centre wells. The gas phase in the manometers was air, and they were equilibrated at 25° for 10–20 min., and the contents of the side arms mixed with those of the main compartments. Readings were taken at 10 min. intervals and recorded.

Incorporation of [^{14}C] *acetate by whole yeast cells*

The methods used were based on those described by Kornberg (1958).

Yeast cells which had been centrifuged after growth in Fernbach culture flasks were washed, and re-suspended in 0·9 % (w/v) KCl at a concentration equiv. *c.* 10 mg. dry wt./ml. About 50 ml. of the suspension were shaken in a Fernbach flask at 25° for about 1 hr. to lower the rate of endogenous respiration.

Of this suspension, 7 ml. were added to 7 ml. of 0·2 M-phosphate buffer (pH 5·9) in the 100 ml. conical Quickfit flask of an automatic pipette. (These pipettes, delivering 1 ± 0·1 ml., can be obtained from W. G. Flaig and Sons Ltd., 39 Waterloo

Road, London, N.W. 2.) The pipette flask was partly immersed in a Warburg bath and shaken at 25°.

Two Warburg manometers, operating in the same bath, were used to follow, simultaneously, the rates of respiration of the yeast, one with and the other without substrate. The Warburg flasks (approx. vol. 20 ml.), with single side arms, contained 1 ml. yeast suspension and 1 ml. 0·2 M-phosphate buffer (pH 5·9) in the main compartments; and one flask had 25 μmole sodium acetate in 0·1 ml. of water in the side arm. Strips of Whatman no. 40 filter paper and 0·2 ml. of 2N-KOH were placed in the centre wells. The gas phase in the manometers was air, and they were equilibrated for about 20 min. at 25°. Readings were taken at 5 min. intervals and recorded.

The contents of the Warburg flask side arm were mixed with those of the main compartment, and at the same moment 175 μmole of sodium acetate in 0·5 ml. water were added to the pipette flask and the contents well mixed.

When the rate of acetate oxidation was steady (usually after about 20 min.) the automatic pipette was removed from the bath, 0·5 ml. [1:2-^{14}C] acetate solution (prepared by mixing equal portions of [1-^{14}C] and [2-^{14}C]acetate, and containing 18·5 μmole of acetate, 100 μc., and giving $6{\cdot}5 \times 10^6$ counts/min. under the conditions of radio-assay used) was added and the top of the pipette replaced. After rapid manual swirling of the contents to ensure good mixing, a 1 ml. sample was pipetted into a centrifuge tube standing in ice. At intervals after adding the radioactive acetate further samples were removed and the time was recorded to the nearest second. Each sample was centrifuged immediately at *c.* 20,000g at −3° for *c.* 30 sec. and the supernatant fluids discarded. (These supernatant fluids did not contain a significant quantity of ethanol-soluble and non-volatile radioactive material. This procedure eliminated interference from radiation decomposition products; the latter, forming 0·05–0·1 % of the total radioactivity of acetate (cf. Kornberg, 1958), evidently did not penetrate the cells.)

Preparation of aqueous ethanolic extracts. To the packed cells was rapidly added 1 ml. of absolute ethanol; the suspension was stirred well and re-centrifuged. The clear supernatant solutions were poured into thick-walled Pyrex tubes and the precipitates were resuspended, each in 1 ml. 20 % (v/v) ethanol in water. After re-centrifuging the corresponding supernatant solutions were combined and evaporated to dryness at *c.* 60° under a stream of nitrogen. The dry material was resuspended in 0·5 ml. of 20 % (v/v) ethanol in water, and samples thereof (0·1 or 0·2 ml.) were analysed by chromatography and radioautography as previously described (Kornberg, 1958).

Experiments with cell-free extracts

Preparation of extracts. Frozen cells were crushed in a Hughes Press (Hughes, 1951) without using abrasive. The crushed yeast was mixed with 9 vol. of 0·9 % (w/v) KCl and homogenized in a glass homogenizer (Umbreit, Burris & Stauffer, 1949). The homogenate was centrifuged at *c.* 20,000g at −3° for 5 min., and the supernatant solution used for the experiment.

The acetic thiokinase activity of the supernatant solutions was measured by the method of Jones & Lipmann (1955).

Malate synthetase activity. In early experiments this was measured as the quantity of isotope incorporated into malate when cell extracts were incubated with [^{14}C]acetate, $MgCl_2$, glutathione, coenzyme A, adenosine triphosphate (ATP) and glyoxylate (Kornberg & Madsen, 1958; see below). In later experiments, the activity of the enzyme was measured by the optical procedure of Dixon & Kornberg (1959).

Iso*citratase activity* was determined by the assay of glyoxylate (Friedemann & Haugen, 1943) formed by the cleavage of *iso*citrate (see Kornberg & Madsen, 1957), the identity of the keto compound being confirmed by chromatography of the 2:4-dinitrophenylhydrazones (El Hawary & Thompson, 1953). The development of more direct assay procedures (Dixon & Kornberg, 1959) allowed quantitative determinations to be made during later parts of this work.

Incorporation of [^{14}C]*acetate by cell-free extracts.* The methods used for studying the incorporation of labelled acetate by cell-free extracts were modified from those described by Kornberg & Madsen (1958). The incubation mixture contained 100 μmole potassium phosphate (pH 7·5), 10 μmole reduced glutathione, 10 μmole $MgCl_2$, 10 μmole ATP, 0·08 μmole coenzyme A (Sigma Chemical Co., St Louis, Missouri, U.S.A.), 0·1 ml. of crushed yeast extract, 1·9 μmole $CH_3{}^{14}COONa$ (containing 10 μc. ^{14}C; giving $6{\cdot}5 \times 10^5$ counts/min.), 10 μmole unlabelled potassium acetate. The quantities of other unlabelled substrates, added as indicated, were as follows: 10 μmole sodium oxaloacetate; 10μmole sodium glyoxylate; 40 μmole DL + allo *iso*citrate (potassium salt); 20 μmole sodium pyruvate; $0{\cdot}4 \times 10^{-3}$ μmole diphosphopyridine nucleotide. Water was added to 1 ml. After incubation at 30° for 30 min., the reaction in each tube was stopped by adding 3 ml. hot absolute ethanol. The 75 % (v/v) ethanolic aqueous suspensions were centrifuged at *c.* 250 **g**, the precipitates washed with 1 ml. 20 % (v/v) ethanol in water and the combined supernatant solutions evaporated to dryness at *c.* 60° under a stream of nitrogen. The chromatographic-autoradiographic analysis was carried out as described by Kornberg (1958).

Protein estimation. The protein content of each yeast extract was estimated by nephelometric-turbidity measurements of trichloro-acetic acid precipitates (Stadtman, Novelli & Lipmann, 1951). A standard curve was obtained by using Armour's bovine serum albumin as a standard. For the estimations, 0·2 ml. of the yeast extract was added to 1·8 ml. water; to this 3 ml. of 5 % (w/v) trichloro-acetic acid was added, and the turbidity measured.

RESULTS

Growth experiments

The results for the K + yeasts are summarized in Table 1. The yeasts listed below were found unable to grow on citrate, fumarate, malate or succinate as sole sources of carbon. The response to acetate, where tested, is indicated in brackets: [+] growth, [−] no growth. *Kloeckera apiculata* (LTS 5); *Saccharomyces acidifaciens* (CBS 749, DEDR 427, ATCC 8766, CBS 685, NRRL Y54, ATCC 2602, CBS 1085); *S. bailii* (CBS 680 [+]); *S. bisporus* (CBS 702 [+]); *S. cerevisiae* (NCYC 77 [+], NCYC 231 [+], NCYC 176 [+], CARL 53, LTS 4); *S. cerevisiae* var. *ellipsoideus* (NCYC 93 [+], NCYC 94 [+], CBS 818 [−]); *S. delbrueckii* var. *mongolicus* (CBS 705); *S. fermentati* (NCYC 161); *S. mellis* (CBS 736, CBS 684, DBDR Z7, CBS 737, CBS 742, DBDR M1); *S. pastori* (CBS 704); *S. rosei* (CBS 728); *S. rouxii* (LTS 16, LTS 17, LTS 18, CBS 710, CBS 731, CBS 732, ATCC 2623, CBS 682, CBS 687, CBS 688, CBS 686, DBDR *Z*5, CBS 738, CBS 741, ATCC 4898, DBDR *Y*139, ATCC 2619, DBDR *Z*1); *S. rouxii* var. *polymorphus* (CBS 711, DBDR *Z*3, NCYC 170, DBDR *Z*4, DBDR 58, CBS 678, CBS 706, CBS 734, ATCC 2624, DBDR *Z*2, CBS 727, CBS 713, CBS 726, ATCC 2617). These data suggest the following two generalizations. First, K+ yeasts (capable of utilizing TCA cycle intermediates) include those tested which Wickerham (1955; Wickerham & Burton, 1956*a*, *b*)

Table 1. *Results of growth tests on TCA cycle intermediates as substrates: K+ yeasts*

Auxanograms. For each auxanogram, a suspension of *c.* $2{\cdot}5 \times 10^8$ yeast cells in 0·5 ml. of water was added to a tube containing 10 ml. melted agar medium without carbon source, at 48°. After mixing, the medium was poured into a Petri dish, cooled, and the surface dried in an incubator. Test carbon sources were placed on the agar surface of each plate, which was incubated and examined every 2 days for a week. T-*tubes.* Each T-tube contained initially 10 ml. of carbon-free liquid medium, 1 % (w/v) of carbon source, yeast cells to give an initial nephelometer reading ('zeroed' at 52) of *c.* 10. The T-tubes were shaken at 25° and nephelometer readings taken at 0, 2, 4 and 6 days. *Symbols.* The letter '*A*' indicates that the auxanographic method was used: in all other cases T-tubes were employed. C = citrate; F = fumarate; M = malate; S = succinate; Ac = acetate. − <10, + 10–20, ++ >20 (difference between initial and final nephelometer reading). The auxanographic responses are given in terms of + (growth) and − (no growth). nt = not tested.

			Substrate				
CBS name	Strains		C	F	M	S	Ac
Candida krusei	CBS 2066		++	++	++	++	++
C. utilis	NCYC 359	*A*	+	+	+	+	nt
Debaryomyces kloeckeri	LTS 1		++	++	++	++	−
Hansenula anomala	CBS 1981, CBS 2576, CBS 2577		++	++	++	++	++
H. suaveolens	NCYC 57		−	++	++	++	++
Pichia membranaefaciens	NCYC 54, CBS 240	*A*	−	+	−	+	nt
Rhodotorula flava	NCYC 69		−	++	+	+	−
Saccharomyces drosophilarum	CBS 2896		++	++	++	++	++
S. fermentati	CBS 748	*A*	−	+	−	+	nt
			−	−	+	++	−
S. florentinus	CBS 746, NCYC 172	*A*	−	+	−	+	nt
S. lactis	CBS 739		−	+	−	+	nt
			−	−	−	++	++
	CBS 683	*A*	−	+	−	+	nt
			−	−	++	++	nt
	CBS 743	*A*	−	+	−	+	nt
			−	−	−	+	++
S. marxianus	NCYC 111		+	+	+	++	++
	NCYC 243	*A*	−	+	−	+	nt
			−	+	++	++	++
	CBS 745	*A*	−	+	−	+	nt
			+	−	+	+	nt
	CBS 712	*A*	+	+	+	+	nt
			++	+	++	++	nt
S. pini	CBS 744		++	+	+	++	−

placed in the group 'Dekkeromyces' or Kudriavzev (1954) in the group 'Fabospora' (and 'Zygofabospora'); these were *Saccharomyces drosophilarum*, *S. lactis*, and *S. marxianus*. Secondly, K-yeasts include *S. acidifaciens*, *S. cerevisiae* and some of the Saccharomyces most able to tolerate high concentrations of sugar, such as *S. mellis* and *S. rouxii* (e.g. Ingram, 1958). In these experiments, all the K+ yeasts were capable of using succinate; of the other TCA cycle intermediates, citrate was used least often. A minority of K+ and K− strains did not grow on acetate.

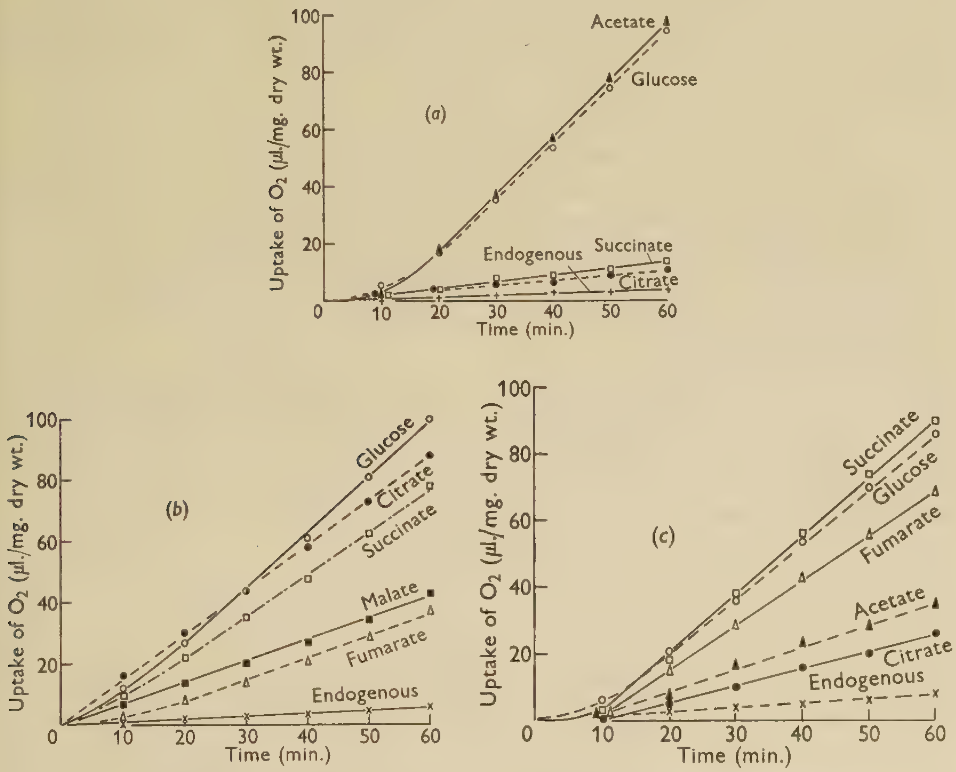

Fig. 1. Oxidation of tricarboxylic acid cycle intermediates by *Saccharomyces marxianus* (CBS 712) grown on (*a*) acetate, (*b*) citrate, and (*c*) succinate as the sole source of carbon. Manometer flasks contained: washed yeast in 2 ml. of 0·1 M-phosphate buffer (pH 5·9) in the main compartments; 25 μmole substrate (as indicated) in 0·1 ml. water in the side bulbs; 0·2 ml. of 2 N-KOH with filter paper in each centre well. Incubated in air at 25°; substrates added at zero time.

Manometric experiments

The results of the measurements of the oxidation of TCA cycle intermediates are summarized in Table 2. Curves showing the course of oxidation in certain cases are given in Fig. 1. These experiments showed that a number of yeasts of different genera were capable of respiring acetate and intermediates of the TCA cycle at rates comparable to that at which they oxidized glucose. Yeasts grown on acetate tended to respire TCA cycle intermediates only very slowly, as compared with those cultivated on succinate. Two strains, *Saccharomyces cerevisiae* (NCYC 231) and

S. acidifaciens (CBS 749), which did not grow on TCA cycle intermediates, were also unable to respire citrate, succinate or fumarate.

Thus, generally, yeasts able to grow on TCA cycle intermediates could respire them too, and conversely. A case of special interest, therefore, is the behaviour of *Saccharomyces drosophilarum*, CBS 2896, which had been grown on citrate as sole carbon source. These citrate-grown cells, unlike those of *S. marxianus*, CBS 712, not only respired other TCA cycle intermediates particularly slowly, but seemed unable to respire citrate itself (Table 2). When washed citrate-grown cells were resuspended in citrate medium of the kind in which they had been grown, the rate of respiration was indistinguishable from that of the negative-control cells to which no substrate had been added. However, a growing 12 hr. culture, concentrated by centrifugation

Table 2. *Rates of respiration on TCA cycle intermediates as substrates*

Manometer flasks contained washed yeast cells in 2 ml. of 0·1 M-phosphate buffer, pH 5·9, in the main compartments; 25 µmole of substrates (as indicated) in 0·1 ml. of water in the side bulbs; and 0·2 ml. of 2 N-KOH with filter paper in each centre well. Incubated in air at 25°. The figures for Q_{O_2} have not been corrected for endogenous respiration. 24 hr. cultures were used, unless otherwise indicated. Abbreviations: G = glucose; Ac = acetate; C = citrate; S = succinate; F = fumarate; M = malate; END = no carbon substrate.

		Q_{O_2}: Uptake of oxygen (µl./mg. dry wt. of yeast/hr.)						
Yeast	Growth medium	G	Ac	C	S	F	M	END
Saccharomyces drosophilarum CBS 2896	Y-M	174	110	15	44	7	28	5
		148	51	12	68	8	8	6
	A_2 glucose	38	.	4	11	4	2	3
	A_2 acetate	74	66	11	3	4	.	4
		128	130	41	24	17	.	15
	A_2 citrate	69	61	9	14	9	.	6
		86	48	7	17	.	12	7
	A_2 succinate	103	.	10	173	58	107	4
		136	67	17	77	34	.	12
S. marxianus CBS 712	Y-M	111	.	14	14	14	14	11
		104	.	.	19	15	15	13
	A_2 glucose	112	.	.	29	10	49	4
		78	.	23	14	9	3	4
	A_2 acetate	125	125	10	13	7	.	5
		88	87	25	11	.	20	7
	A_2 citrate (48 hr.)	116	.	91	98	45	46	5
	A_2 succinate	150	155	134	166	158	120	7
		140	.	133	134	128	112	9
Candida krusei CBS 2066	Y-M	64	76	17	30	13	17	13
	A_2 succinate	11	4	2	24	10	.	2
	A_2 acetate	33	.	5	10	5	.	6
Hansenula anomala CBS 1981	Y-M	97	78	43	31	15	15	9
	A_2 acetate (48 hr.)	13	24	2	1	1	.	2
	A_2 succinate	31	10	30	26	17	.	6
Saccharomyces cerevisiae NCYC 231	A_2 acetate	102	77	4	3	3	.	3
S. acidifaciens CBS 749	A_2 acetate	90	78	4	6	5	.	6

to equiv. 2·7 mg. dry wt. yeast/ml. A_2Cit. medium (but the cells not washed, and the medium not changed), gave a Q_{O_2} of 150 and an R.Q. of 1·7. A comparable situation was reported by Cabelli (1955) for *Klebsiella pneumoniae.* It is possible that washing the cells impaired their ability to oxidize citrate through the removal of a necessary co-factor.

Incorporation of [1:2-^{14}C]*acetate by whole cells*

In six experiments, the incorporation of ^{14}C from labelled acetate by whole cells was studied. Of these experiments, two were carried out with succinate-grown *Saccharomyces drosophilarum* (CBS 2896), one with this yeast grown on acetate, one with it grown on citrate, and one each with *S. cerevisiae* (NCYC 231) and *S. acidifaciens* (CBS 749) grown on acetate. The results of experiments performed under comparable conditions were similar; those obtained with *S. drosophilarum* are shown in Fig. 2. After addition of labelled acetate to the respiring yeast, ^{14}C was rapidly and linearly incorporated into the fractions soluble in aqueous ethanol. At the earliest times, isotope appeared only in intermediates of the TCA cycle and in amino acids derived from them. The labelling patterns (Fig. 2*a*) observed with the yeast grown on succinate (and citrate) are consistent with those expected from the operation of the TCA cycle. This is not so for the acetate-grown yeasts (Fig. 2*b*). In particular, the proportions of isotope present in succinate and malate indicate that, at the earliest times, ^{14}C from acetate entered malate without passing through the stage of succinate. Whilst, during the later part of the experiment, succinate contained significantly more ^{14}C than did malate (indicating that the 'pool size' of succinate was greater than than of malate), the succinate in the earliest samples contributed less to the total radioactivity than did malate. The ratio ^{14}C incorporated into malate: ^{14}C incorporated into succinate rose with time in the succinate-grown yeast, which is consistent with the expected formation of labelled malate from labelled succinate (Table 3), but decreased with time in acetate-grown cells. This indicates that, in the acetate-grown cells, labelled succinate could not have been the sole precursor of labelled malate, but that pathways must operate whereby ^{14}C from acetate can enter malate without passing first through succinate.

These results, which are similar to those reported for *Pseudomonas fluorescens* (Kornberg, 1958), offer presumptive evidence that acetate is utilized by the acetate-grown yeast via the concerted action of the tricarboxylic acid and glyoxylate cycles (see Kornberg & Krebs, 1957), but that this latter cycle does not operate in yeasts grown on succinate.

Experiments with cell-free extracts

Measurements of acetate-activation. The results of assaying the activity of acetic thiokinase in extracts of the yeasts are shown in Table 4. The extracts from *Saccharomyces drosophilarum* (CBS 2896) grown on acetate or succinate, and *S. acidifaciens* (CBS 749) and *S. cerevisiae* (NCYC 231) grown on acetate were each capable of forming about 2 μmole acethydroxamic acid/mg. protein/hr. from acetate under the test conditions of Jones & Lipmann (1955).

Measurements of iso*citratase activity.* Measurements of the rate of glyoxylate production from *iso*citrate in extracts from the same yeasts are shown in Table 5. *Saccharomyces drosophilarum* (CBS 2896) grown on acetate as sole carbon source

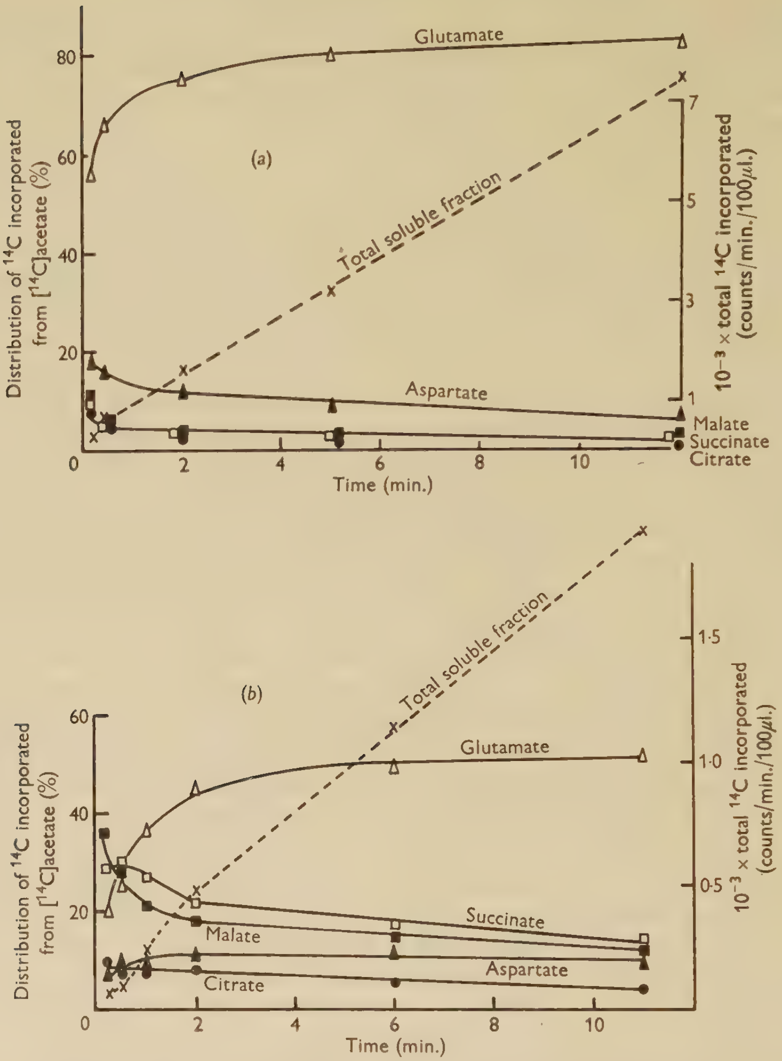

Fig. 2. Incorporation of ^{14}C from [1:2-^{14}C]acetate into the ethanol-soluble fractions of *Saccharomyces drosophilarum* (CBS 2896) respiring acetate: (*a*) succinate-grown, (*b*) acetate-grown. A solution of $^{14}CH_3.^{14}CO_2Na$, containing 18·5 μmole acetate and 100 μc. of ^{14}C, was added at zero time to 14 ml. of a suspension of the succinate- or acetate-grown yeast, respiring in an automatic pipette on 12·5 mM-sodium acetate. Samples (approx. 1 ml.) were pipetted at known times into tubes standing in ice. The precipitates were each treated with 1 ml. of absolute ethanol, and each precipitate washed with 1 ml. of 20 % (v/v) aqueous ethanol. The corresponding supernatant solutions were combined and evaporated to dryness under N_2, resuspended in 20 % (v/v) aqueous ethanol and analysed by two-dimensional paper chromatography. The radioactive compounds were located by radioautography, and the ^{14}C content was determined by direct radioassay of the radioactive areas with a mica end-window β-counter tube. Each point on the graphs represents the mean of two chromatograms.

Table 3. *Change with time of the ratio of the amount of isotope in succinate to that in malate for respiring suspensions of acetate- and succinate-grown* Saccharomyces drosophilarum (*CBS* 2896) *incubated with sodium* [1:2-^{14}*C*]*acetate*

Conditions were similar to those described in Fig. 2.

Carbon source for growth of suspensions	Time (sec.)	Ratio: $\frac{^{14}\text{C incorporated into malate}}{^{14}\text{C incorporated into succinate}}$
Succinate	9	1·0
	24	1·1
	120	0·81
	300	1·3
	737	1·6
Acetate	10	1·2
	30	1·0
	60	0·82
	120	0·78
	360	0·94
	660	0·84

Table 4. *Acetic thiokinase activity of cell-free extracts*

Each tube contained 350 μmole potassium phosphate (pH 7·5), 35 μmole $MgCl_2$, 36 μmole reduced glutathione, 170 μmole KF, 35 μmole ATP, 700 μmole hydroxylamine, 70 μmole potassium acetate, 0·28 μmole coenzyme A, 0·5 ml. of yeast extract, and water to 3·5 ml. Incubation was at 30° for 30 min., 1 ml. samples pipetted into 1·5 ml. of $FeCl_3$ reagent, and the acethydroxamate measured with 1 cm. glass cells in an Unicam SP. 600 at 540 mμ.

Yeast	Carbon source for growth of cells	Activity of extract (μmoles acethydroxamic acid/mg. protein/hr.)
Saccharomyces drosophilarum (CBS 2896)	Acetate	2·4
	Succinate	2·4
	Citrate	0·2
S. acidifaciens (CBS 749)	Acetate	2·4
S. cerevisiae (NCYC 231)	Acetate	2·2

readily formed glyoxylate from *iso*citrate, the specific activity of the enzyme in the crude cell extract was 15·5 μmole glyoxylate formed/mg. soluble protein/hr., as estimated by the method of Dixon & Kornberg (1959). The corresponding specific activity for extracts of succinate-grown cells was 0·56, for those grown on citrate 0·55. Acetate-grown *S. acidifaciens* (CBS 749) and *S. cerevisiae* (NCYC 231) gave an activity of a half and a quarter, respectively, of that of acetate-grown *S. drosophilarum.*

These differences in the measured rates of glyoxylate formation from *iso*citrate by extracts obtained from *Saccharomyces drosophilarum* grown on succinate, citrate or acetate as carbon sources, were also strikingly confirmed by visual examination of the 2:4-dinitrophenylhydrazones of the keto acids formed under these conditions (Fig. 3). All the extracts from *S. drosophilarum* produced, from added *iso*citrate, α-ketoglutarate and a small quantity of pyruvate; only the extract of the acetate-grown yeast, however, catalysed in addition the formation of appreciable amounts of glyoxylate.

Measurements of malate synthetase activity. Extracts from *Saccharomyces drosophilarum* (CBS 2896) grown on acetate had a malate synthetase activity under the conditions of assay of 16·8 μmole acetyl CoA split/mg. soluble protein/hr. The corresponding figures for extracts of the yeast grown on succinate and citrate were 4·3 and 4·2 (Table 5).

Table 5. Iso*citratase and malate synthetase in extracts of* Saccharomyces drosophilarum (*CBS* 2896)

Extracts were prepared by crushing the yeast, grown on acetate, succinate or citrate as sole carbon sources, in a Hughes press, and homogenizing the crushed material with equal volumes of 0·1 M-potassium phosphate, pH 7·5. To each ml. of these suspensions were added 0·1 ml. of 2 % protamine sulphate. The precipitates were removed by centrifugation. The iso*citratase activity* of the clear supernatant solution was estimated as the rate of change of optical density at 324 mμ when the extracts were incubated with 200 μmole potassium phosphate, pH 6·9, 6 μmole cysteine hydrochloride, 20 μmole magnesium chloride, 5 μmole phenylhydrazine hydrochloride, 5 μmole potassium L_s-*iso*-citrate and water to 3 ml. *Malate synthetase* was determined by the rate of decrease of optical density at 232 mμ resulting from the enzymic removal of acetyl coenzyme A in the presence of glyoxylate (Dixon & Kornberg, 1959). The ratios *A* and *B* were calculated from the data in Table 6, ratio *C* from those in this table.

Carbon source for growth	Protein concn. of original extract (mg./ml.)	Specific activity (μmoles reacting/mg. protein/hr.) of		Ratio		
		*iso*Citratase	Malate synthetase	*A* Condensing enzyme / Malate synthetase	*B* Condensing enzyme / *iso*Citratase	*C* Malate synthetas / *iso*Citratase
Acetate	18	15·5	16·8	0·85	1·13	1·08
Succinate	14·8	0·56	4·3	2·47	34·8	7·7
Citrate	28	0·55	4·2	2·30	33·2	7·6

Incorporation of [^{14}C]*acetate.* The results of experiments on the reactions of [^{14}C]acetate in extracts are given in Table 6. The assay showed that extracts of the yeast grown under all conditions promoted the incorporation of major amounts of label from [^{14}C]acetate into citrate, in the presence of oxaloacetate, and into malate, in the presence of glyoxylate. However, the incorporation into malate, in the presence of *iso*citrate, was much less in the succinate and citrate-grown yeast than in that grown on acetate.

DISCUSSION

The ability of K+ *yeasts to use succinate.* A survey of the literature suggests that most K+ yeasts can use succinic acid: that is, few yeasts seem unable to use succinate, but able to grow on citrate, fumarate or malate. Exceptions have been reported for *Pichia farinosa*, a strain of a Torulopsis species (Beech, 1957), *Torulopsis pseudaeria* (Zsolt, 1958), *Rhodotorula crocea* (Shifrine & Phaff, 1956) and *Saccharomycopsis guttulata* (Shifrine & Phaff, 1958). All the K+ yeasts studied for the present work (Table 1) utilized succinate. However, the degree of agreement in the literature is more surprising than any disagreement. Apart from expected differences between strains, the answers obtained seem to depend greatly on the methods used, as is indicated by the results for *Saccharomyces fermentati*, *S. lactis*, and *S. marxianus* shown in Table 1. None the less previously published work supports the tentative generalizations already made about the 'Dekkeromyces' group and *Saccharomyces* *mellis* and *S. rouxii* (cf. Albritton, 1953; Barnett & Ingram, 1955; Beech, 1957; Hedrick & Burke, 1951; Miller & Phaff, 1958; Nickerson & Carroll, 1945; Niehaus, 1932; Phaff & Knapp, 1956; Phaff, Miller & Shifrine, 1956; Shehata, Mrak & Phaff, 1955; Shifrine & Phaff, 1956; Skinner & Huxley, 1956; Teunisson, 1954; Wiles, 1954).

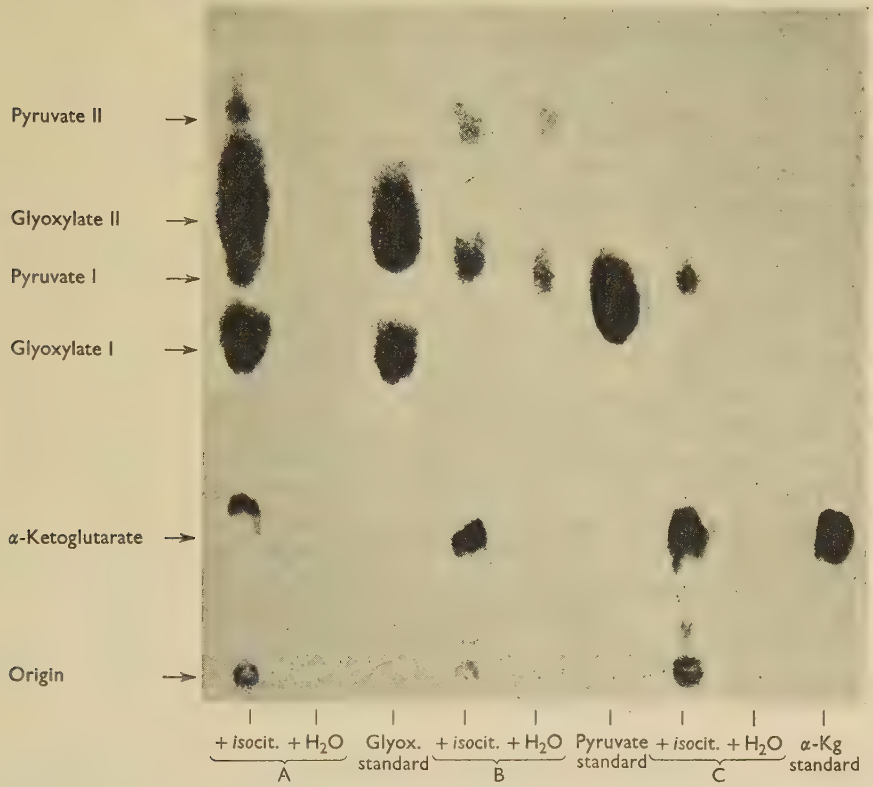

Fig. 3. Chromatogram of the 2:4-dinitrophenylhydrazones of the keto acids produced by extracts of *Saccharomyces drosophilarum* (CBS 2896) incubated alone and with *iso*citrate. Cells grown on acetate (A), citrate (B) or succinate (C) as sole carbon sources.

The characterization of Saccharomyces cerevisiae. Of great interest are the yeasts classified as *S. cerevisiae*, *S. carlsbergensis* and similar organisms. They are of considerable industrial importance and there is much biochemical literature on the terminal respiratory mechanisms of *S. cerevisiae*, usually as baker's yeast. There is much confusion about the biochemical characteristics of *S. cerevisiae*, and it is worth reviewing the facts at some length here. Baker's yeast is almost always *S. cerevisiae* (White, 1954; Nickerson, 1957; Pyke, 1958). Brewer's yeast is commonly so, though lager yeasts may be classified as *S. carlsbergensis* (Guilliermond, 1920; Lodder & Kreger-van Rij, 1952; Thorne, 1957). The data given earlier agree with those of Kudriavzev (1954) in showing that *S. cerevisiae* is unable to grow on exogenous intermediates of the TCA cycle. This is also in agreement with observations on baker's and brewer's yeasts by other workers (e.g. Brandt, 1945; Krebs,

Table 6. *Reactions of* [^{14}*C*]*acetate in extracts of yeasts*

Extracts were prepared by crushing the yeast, grown on either acetate, succinate or citrate, in a Hughes Press and homogenizing the crushed material with 9 vol. of 0·9 % KCl. The suspensions were centrifuged at 15,000 *g* and 0·1 ml. of the supernatant solutions incubated with potassium phosphate, $MgCl_2$, ATP, coenzyme A, glutathione, [^{14}C]acetate and additional substrates as indicated, at 30° for 30 min. At this time, 3 ml. hot absolute ethanol was added to each tube, and the ethanol soluble fractions were subsequently analysed by chromatography and autoradiography.

	Carbon source for growth of yeast	Protein concentration of original extract (mg./ml.)	Substrate added	Radioactivity (counts/min.) incorporated into	
				Malate	Citrate
Saccharomyces drosophilarum (CBS 2896)	Acetate	5·46	None	40	20
			Oxaloacetate	60	159,000
			*iso*Citrate	140,000	30
			Glyoxylate	187,000	10
	Succinate	7·27	None	20	10
			Oxaloacetate	40	160,000
			*iso*Citrate	4,660	60
			Glyoxylate	64,800	60
	Citrate	9·05	None	20	10
			Oxaloacetate	60	89,100
			*iso*Citrate	2,690	30
			Glyoxylate	38,800	30
S. acidifaciens (CBS 749)	Acetate	2·41	None	70	40
			Oxaloacetate	650	189,000
			*iso*Citrate	165,000	70
			Glyoxylate	194,000	40
S. cerevisiae (NCYC 231)	Acetate	1·92	None	60	20
			Oxaloacetate	180	105,000
			*iso*Citrate	122,000	90
			Glyoxylate	151,000	40

1949; Barron, Ardao & Hearon, 1951; Slonimski, 1953; Krebs, 1954; Singer, Massey & Kearney, 1957). An exception is the report by Krebs (1935) of baker's yeast respiring α-ketoglutarate. Kilkenny & Hinshelwood (1951) reported that five strains of *S. cerevisiae* (designated A, B, C, D, E) were able to grow on all the acids of the TCA cycle, except for strain C which did not grow on aconitic, citric, α-ketoglutaric or oxaloacetic acids. Probably none of these five was in fact a strain of *S. cerevisiae* (Sir C. Hinshelwood, personal communication 1957). They were identified later at the Yeast Division of the CBS (Dr W. Ch. Slooff, personal communication 1957) as three strains of *Hansenula anomala* (A, B and D), one of *Candida krusei* (C) and one (E) of which the identity is in doubt. It would be more useful to regard *S. cerevisiae* as a K – yeast, and all strains capable of metabolizing exogenous TCA cycle intermediates would thus be excluded from this species. This would agree with the exclusion of *Debaryomyces mandshuricus* Naganishi, by Kudriavzev (1954) and of *Zygosaccharomyces fermentati* by both Kudriavzev (1954) and Phaff, Miller & Shifrine (1956). These two were classified as *S. cerevisiae* by Lodder & Kreger-van Rij (1952).

The tricarboxylic acid cycle in yeasts. The growth studies described here, and those of previous workers, show that yeasts can be classified as K+ or K− by their ability to utilize for growth intermediates of the TCA cycle, but this classification does not imply that K− yeasts lack this cycle. Many K+ yeasts cannot utilize all the cycle intermediates. Manometric data also differentiate K+ and K− yeasts, but the oxidation of intermediates is variable and depends on the conditions of growth. Moreover, K+ yeasts grown on acetate tend to have the respiratory behaviour of K− yeasts.

These findings can be explained by the existence of permeability barriers, which prevent free access of externally added substrates to intracellular enzyme systems. All the component reactions of the TCA cycle have been shown to occur in extracts of acetate-grown yeasts (for review, see Kornberg, 1959*a*), and the cycle has been shown to be of major importance in acetate oxidation by baker's yeast, a typical K− yeast (De Moss & Swim, 1957). Comparison of the patterns of isotope incorporation from [1:2-^{14}C]acetate by a K+ yeast (*Saccharomyces drosophilarum*) with those of K− yeasts (*S. cerevisiae*, *S. acidifaciens*) support this view. Although the procedure used in this work does not permit quantitative estimates to be made of the rate of incorporation of isotope from acetate, the distribution of radioactivity among the labelled components of each sample fairly reflects the time course of acetate incorporation. It is therefore significant that isotope from acetate was incorporated at early times only into intermediates of the TCA cycle and into amino acids (such as aspartate and glutamate) directly derived from them, and that these labelled compounds are found both in K+ yeasts grown on succinate or citrate (which presumably metabolize these materials and acetate via the TCA cycle) and in acetate-grown K− yeasts. Metabolism of acetate via the TCA cycle would result in an isotope content of succinate initially higher than that of malate: this was found to be the case (Table 3). Only in acetate-grown yeasts was this criterion not obeyed, and malate acquired isotope from acetate without passing through the stage of succinate. This indicated the operation of the glyoxylate cycle (Kornberg & Krebs, 1957) under these conditions, in addition to that of the TCA cycle.

The glyoxylate cycle in yeasts. The key enzymes of the glyoxylate cycle are *iso*citratase (Smith & Gunsalus, 1955; Olson, 1954) and malate synthetase (Wong & Ajl, 1956), both of which have been shown to be present in extracts of baker's yeast (Olson, 1959; Kornberg, 1959*b*). The experiments reported in this paper confirm these findings. Extracts of acetate-grown yeasts, supplemented with necessary cofactors, catalysed the incorporation of isotope from acetate into malate if incubated with glyoxylate or *iso*citrate (Table 6), and contained high activities of malate synthetase and *iso*citratase (Table 5). Extracts of yeasts grown on succinate or citrate contained malate synthetase at *c.* 25% and *iso*citratase at *c.* 3% of the specific activities observed with acetate-grown yeasts. This indicates that the operation of the glyoxylate cycle in yeasts may be controlled by influencing the intracellular activity of *iso*citratase, as has also been found with *Pseudomonas ovalis* (Kornberg & Lund, 1959) and *Micrococcus denitrificans* (Kornberg, Collins & Bigley, 1960).

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Some Observations on the Cytochemistry of Streptomyces

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SUMMARY

Tests for the distribution of sulphydryl groups, alkaline phosphatase, catalase, peroxidase in six species of *Streptomyces* showed that these occurred in greatest concentration in the young aerial mycelium, and especially at its points of origin or differentiation from the vegetative mycelium. In some species, these points of differentiation took the form of synnemata (coremia) of a distinctive appearance.

INTRODUCTION

Although a considerable amount of work has been done on the subject (e.g. von Plotho, 1940*a*, *b*; Klieneberger-Nobel, 1947; Erikson, 1947, 1949; Carvajal, 1946, 1947; Gottlieb, 1953; Pénau, Hagemann, Vèlu & Peyré, 1954; Hagedorn, 1955; Romano & Nickerson, 1956; Romano & Sohler, 1956; Sohler, Romano & Nickerson, 1958) our knowledge of the cytology and cytochemistry of actinomycetes is very incomplete. However, considerable differences have been detected between the composition of the primary and secondary mycelia of Streptomyces. It is the purpose of this paper to extend this information in respect of the distribution of sulphydryl groups and of certain enzymes which can be detected with accuracy.

METHODS

Six different strains, all from the collection maintained in this Laboratory, were used: *Streptomyces griseus*, *S. rimosus*, *S. aureofaciens*, *S. chrysomallus*, two unnamed strains, *S.* 829 and *S.* 1594, both of which apparently belong to the same species (but with some different morphological characters) and produce flavensomycin.

Cultures were made on slopes of a medium containing (%, w/v): 0·1, asparagin; 1·0, glycerol; 0·1, K_2HPO_4; 1·8, agar; pH 7·2–7·4. After incubation for 20 days at 27° the spores were used to inoculate strips of sterile cellophan (1·5 × 3 cm.) supported on the surface of plates of the same medium in Petri dishes. Inoculations were made sparsely, so as to produce separate colonies. After periods of 48 hr., 72 hr. and 10 days at 27°, strips were removed and attached to slides with a little gelatin, for cytochemical examination.

Sulphydryl groups were detected by means of the nitroprusside test of Di Mattei & Dulzetto (1928) and by the method of Chèvremont & Fredericq (1943). The colour given by the first test is transient, while that of the second is permanent; it is conducted on material fixed in absolute ethanol or in 20% (w/v) trichloroacetic acid. The latter reagent enhances the intensity of the reaction.

Peroxidase was detected by the method of Spirito (1934), with unfixed films and others treated with absolute ethanol for 20 min. The 'leuco-zinc' method did not give positive results. Alkaline phosphatase was detected by the method of Gomori (1952), with films fixed either in acetone at 4° for 24 hr., or in absolute ethanol at room temperature for 20 min.

Controls were heated to 100° for 10 min. or were incubated without the substrate. Both types of control were used.

Catalase was detected by adding a few drops of '10 vol.' hydrogen peroxide, and observing the result under the microscope. The further addition of a few drops of ethanol was sometimes an improvement (see below).

The purpose of using alternative methods of fixation, and in some instances of staining, was as a control, in case of artifacts caused by the fixation or staining processes themselves.

RESULTS

In making these observations, we also observed very clearly a morphological character which seems to have been described previously only by Krassilnikov (1938). With certain strains of Streptomyces, and only on some media (asparagin glycerol agar for example) the initiation of the aerial mycelium began with the appearance of bundles of hyphae arising from the vegetative mycelium and adhering at the distal ends (Pl. 1, figs. 2, 5), which Krassilnikov designated coremia, although they seem better described as synnemata. These covered the surface of the colony; from their apices the sporogenous hyphae grew outwards (Pl. 1, fig. 3). These were clearly visible even in fresh preparations. With increase of the age of the culture, this appearance vanished and the customary form of the aerial mycelium superseded it. In the present case, these synnemata were produced by all strains examined, except *Streptomyces griseus* and *S. rimosus*.

Sulphydryl groups. At 48 hr. in every case the vegetative mycelium gave a very weak reaction, and the aerial mycelium, especially at its points of origin, whether or not these appeared in the form of synnemata, a much stronger one. After 10 days, the older parts of the aerial mycelium reacted less strongly in most cases, but the younger peripheral hyphae maintained their reactivity. In the case of *Streptomyces aureofaciens*, the whole aerial mycelium continued to react strongly after 10 days (Pl. 1, figs. 1, 2 and 6).

Alkaline phosphatase. After 72 hr. and 10 days the vegetative mycelium gave a weak reaction, and the aerial mycelium a strong one, especially where synnemata were visible (Pl. 1, fig. 3).

Catalase. In all strains examined, bubbles of gas first appeared at the edges of the vegetative mycelium. However, the addition of a few drops of 95% (v/v) ethanol in water to the solution caused immediately a much stronger reaction all over the aerial mycelium. The effect was similar at 48 hr. and 10 days, and was presumably due to the production, by means of the ethanol, of a more intimate contact between the reagent and the lipid-containing surface of the aerial hyphae, which was normally hydrophobic.

Peroxidase. The vegetative mycelium at all stages produced a very slight reaction. The aerial mycelium reacted strongly at 72 hr.; at 10 days, only the young hyphae did so. Areas with synnemata reacted especially strongly, those with mature spores weakly or negatively (Pl. 1, figs. 4, 5).

DISCUSSION

The results show that the aerial mycelium of Streptomyces, especially when young, possesses high concentrations of sulphydryl groups, alkaline phosphatase, catalase and peroxidase. Similar results were obtained with all strains examined. The differences in reaction as between vegetative and aerial mycelium were clearly marked, except in the case of catalase, where the nature of the reaction rendered it less readily attributable to a specific location. However, even in this case, and especially after the addition of ethanol, the difference between the rate of gas production at the centre and that at the edges of the colony was striking enough to suggest that this enzyme also was in much higher concentration in the aerial mycelium as compared with the vegetative mycelium. The exception to the generally weak or negative reaction of the vegetative mycelium to the above reagents was in the growing tips, from which the aerial mycelium was beginning to differentiate. From these results and from those of other workers (e.g. Craveri, Ferrari & Treccani, 1958) it would appear that the greatest metabolic activity is located in the aerial mycelium, and that the vegetative mycelium serves as a support and as a connexion with the culture medium.

So far as the validity of these tests is concerned, the two methods for detecting sulphydryl groups gave the same results. Peroxidase was detectable only by the benzidine method; we believe that the negative results with the 'leuco-zinc' methods were due to their specificity being for haemoglobins rather than for true peroxidases. The controls used for the alkaline phosphatase reaction give reasonable security for its specificity; the heated slides were negative, and those incubated without the specific substrate gave a very weak reaction, attributable to traces of substrate in the culture.

The interesting morphological appearance of synnemata was observable by direct examination and may be influenced by the conditions of growth and by the nature of the substrate; it was, nevertheless, shown only by certain strains, and may accordingly be of value as a taxonomic character.

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EXPLANATION OF PLATE 1

Fig. 1. *Streptomyces griseus*; 48 hr. growth; sulphydryl reaction. Magnification, × 200.

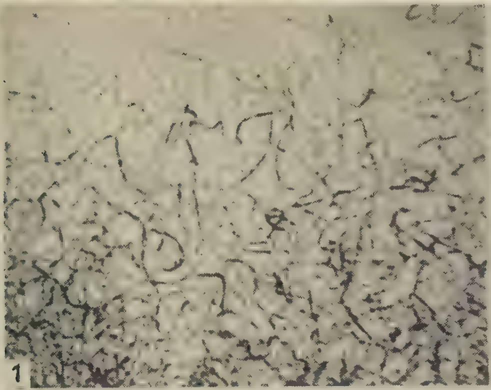
Fig. 2. *Streptomyces* no. 829; 48 hr. growth; sulphydryl reaction. Note the intensity of the reaction of the synnemata. Magnification, × 200.

Fig. 3. As fig. 2; 72 hr. growth; alkaline phosphatase reaction. Note the intensity of the reaction of the synnemata. Magnification, × 200.

Fig. 4. As fig. 1; 72 hr. growth; peroxidase reaction. Magnification, × 200.

Fig. 5. As fig. 2; 72 hr. growth; peroxidase reaction. Note the form of the synnemata and the intensity of their reaction. Magnification, × 200.

Fig. 6. *Streptomyces aureofaciens*; 10-day culture; sulphydryl reaction. Synnemata are intensely stained. Magnification, × 200.



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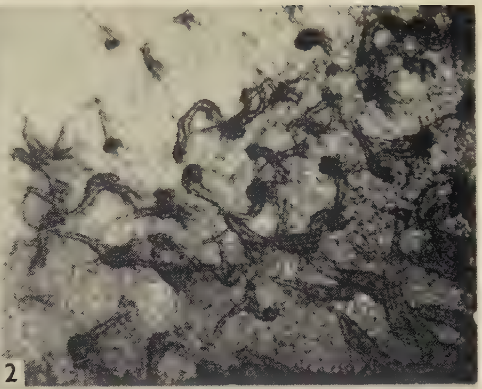

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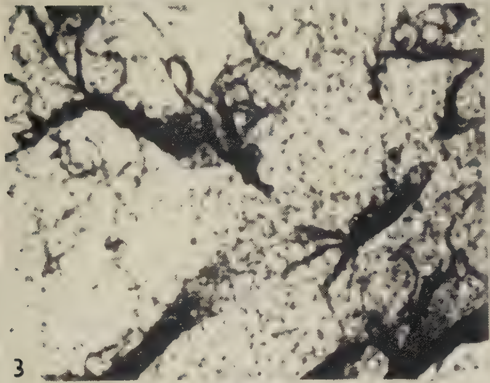

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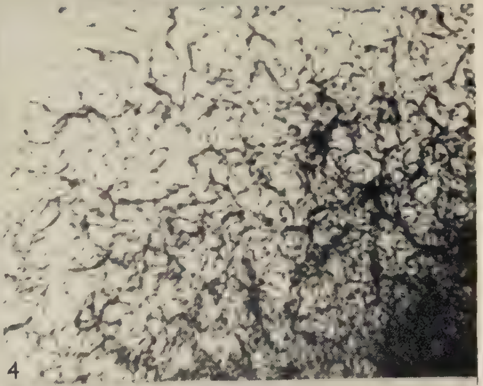

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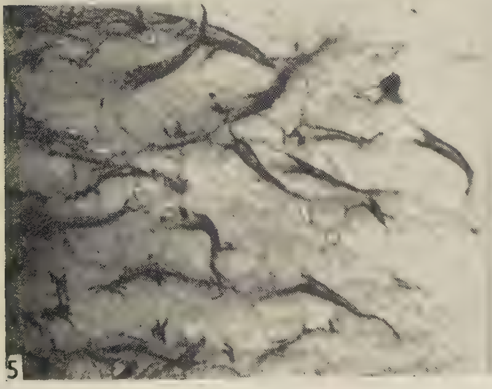

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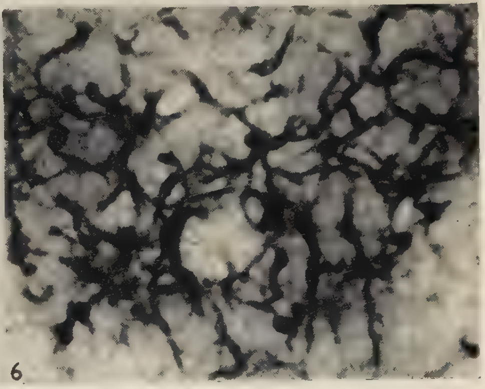

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The Effect of Deficiency of Iron, Zinc and Manganese on the Growth and Morphology of *Nocardia opaca*

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SUMMARY

A study of the trace element requirements of a strain of *Nocardia opaca* was made. Iron, zinc and manganese were required for optimum growth of the organism on glucose, sucrose, glycerol, gluconate and phenylacetate. Deficiency of manganese caused marked morphological changes in the organism during growth on all the substrates.

INTRODUCTION

The trace element requirements of the actinomycetes, as compared with the fungi (see Foster, 1949; Cochrane, 1958) have received little attention in the past. Studies of this nature which have been made have been directed principally towards the elucidation of the trace element requirements of specific Streptomyces strains used for the production of specific antibiotics. Literature dealing with this subject was reviewed by Villemin, Lechevalier & Waksman (1953). Heim & Lechevalier (1956) studied the effect of iron, zinc, manganese and calcium on the growth of eight different strains of Streptomyces. As far as can be ascertained no such study has been made with organisms of the genus *Nocardia*. In the present work it has been found that iron, zinc and manganese are required for optimum growth of a strain of *N. opaca* and also that deficiency of the latter two elements (particularly manganese) causes marked morphological changes in the organism.

METHODS

Organism. Nocardia opaca, strain T_{16}, described previously (Webley, 1954), was used throughout.

Growth experiments. These were carried out in 100 ml. Erlenmeyer or 1 l. culture flasks containing 25 and 200 ml. medium, respectively. All glassware used was of Pyrex brand and was soaked for at least 18 hr. (overnight) in 5 N-HCl before being rinsed many times with double-distilled water obtained from a glass still. The flasks were subsequently drained dry. The basal mineral salts were those of Donald, Passey & Swaby (1952) (g./l.: 5, KNO_3; 2·5, K_2HPO_4; 0·5, $MgSO_4.7H_2O$) with 0·024% (w/v) L-glutamic acid (sodium salt). The substrates used for carbon and energy source were glucose, sucrose, glycerol, sodium gluconate, all at 2% (w/v), and sodium phenylacetate (1%, w/v). The method of treating the basal medium + substrate, to remove as much of the contaminating trace metals as possible, was that of Donald *et al.* (1952) using 2% (w/v) alumina. The basal mineral salts + substrate (except glucose) were adjusted to pH 7·5 and the sodium glutamate solution (to be added separately) to pH 7·8–8·0 (see Heim & Lechavalier, 1956) after addition of the alumina—both solutions being treated separately. When glucose was used, it was also treated separately, and the pH adjusted to 6·8 to avoid excessive breakdown. The strengths of the solutions were made so that when they were finally mixed, and the trace-elements added, the desired final concentrations were obtained. The pH value of the final mixture was adjusted to 7·2 with trace element-free NH_4OH or HCl solutions. After purification, complete medium was obtained by adding to the basal mineral salts + substrate solution (per litre): 200 μg. Fe (as $FeSO_47H_2O$; 180 μg. Zn (as $ZnSO_47H_2O$); 40 μg. Cu (as $CuSO_45H_2O$); 20 μg. Mn (as $MnSO_45H_2O$); 10 μg. Mo (as $(NH_4)_6MO_7O_{24}.4H_2O$); 10 μg. B (as H_3BO_3), unless otherwise stated. The salts used to supply trace elements were spectrographically standardized substances ('Specpure', Johnson, Matthey and Co. Ltd., 73/83 Hatton Garden, London). A specific trace element deficiency was obtained by omitting the salt containing the element in question. The small and large flasks were inoculated with one drop and 0·1 ml., respectively, of a suspension of the organism obtained from a 6-day surface colony on nutrient agar. This suspension was washed 3–5 times with double-distilled water and made finally to contain $2–5 \times 10^5$ viable units/ml. The flasks were incubated at 25° statically or on a reciprocal shaking machine. The organisms were harvested after incubation for 7–12 days and washed 2–3 times with double-distilled water before being resuspended in a known volume. A sample was removed and dried to constant weight at 100° for determination of the total dry weight of cell material.

Morphology and viable counts. Morphological changes were followed by phase-contrast microscopy and by examination of heat-fixed preparations stained with crystal violet. For cell walls the tannic acid-crystal violet technique described by Hale & Bisset (1956) was used. Electron microscopic examination was also occasionally carried out. Viable counts were performed by the method of Miles & Misra (1938).

RESULTS

Effect of deficiency of trace elements on yield of organism and viable counts of Nocardia opaca

Table 1 shows the effect of deficiency of iron, zinc and manganese on the growth of the organism on four different substrates. Iron deficiency produced the greatest effect and the organisms *en masse* were frequently greyish white in colour in contrast to the orange-salmon pink of those grown in complete medium. A similar effect of iron deficiency on pigmentation of *Aerobacter aerogenes* was described by Waring & Werkman (1944). Deficiency of Mn or Zn did not produce this change in pigmentation. The pH value of the iron-deficient medium at the end of the growth period, particularly with glucose as substrate, was always acid (pH 4·0–5·0), in contrast to complete medium and Mn and Zn-deficient media which did not show this effect. The pH values of these cultures rose from the initial pH 7·2 to 8·0–8·5 at the end of the experiments. The omission of Cu, Mo or B did not affect the yields of organism. The addition of 40–400 μg. Ca (as $CaCl_2$; prepared from 'Specpure' $CaCO_3$) per l. complete medium did not markedly affect the growth of the organism. Zn was not replaced by cadmium (as 'Specpure'; cadmium sulphate). In fact, cadmium above 36 μg./l. was toxic to the organism on Zn-deficient medium. Figure 1 shows

Table 1. *Effect of deficiency of iron, manganese and zinc on growth of* Nocardia opaca *on different substrates*

N. opaca grown in 200 ml. complete, or Fe-, or Mn-, or Zn-deficient medium with 2 % (w/v) glucose, sucrose or glycerol or 1 % (w/v) phenylacetate on shaking machine. Organisms harvested after 7 days with glucose or glycerol, and after 10 days with sucrose or phenylacetate, washed 3 times with double-distilled water, made up to known volume and dry weights determined on suitable samples.

Medium	Substrate			
	Glucose	Glycerol	Sucrose	Phenylacetate
	mg. dry wt. organism/100 ml. culture			
Complete	256·0	187·5	146·0	188·0
−Fe	22·8	6·0	11·4	7·2
−Mn	67·5	18·0	64·0	62·5
−Zn	140·0	27·8	51·0	48·0

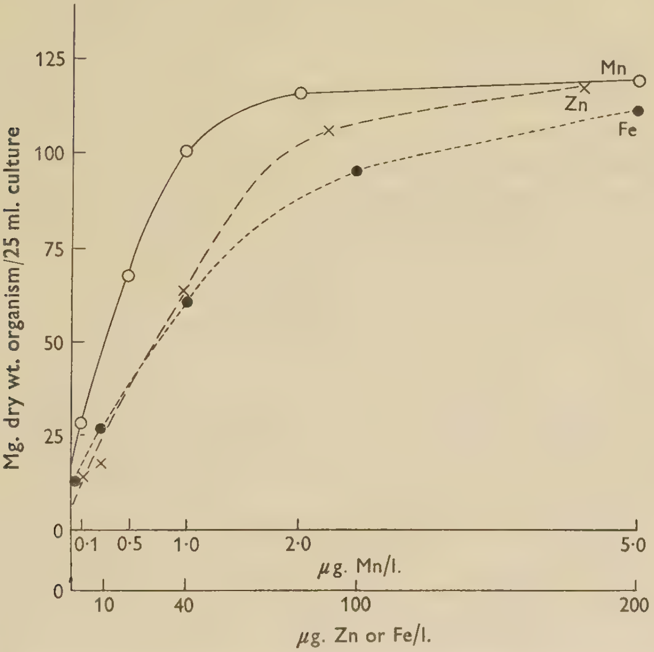

Fig. 1. The relationship between dry weight yields of *Nocardia opaca* and added amounts of Fe, Zn and Mn. The organism was grown in 25 ml. appropriate medium containing 2 % (w/v) glucose for 7 days at 25°. The organisms were harvested and washed three times, made up to a known volume and dry weight determined on a suitable sample.

the growth response of *Nocardia opaca* to different amounts of Mn, Zn and Fe. It is clear that the requirement for Mn was very much smaller than that for Fe and Zn. Table 2 shows the viable counts obtained on the deficient media. Although these figures are not altogether reliable, because of the morphology of the organism, they closely reflect the differences in total yields of organism (see Table 1).

Determinations of the Zn and Mn contents of organisms from the corresponding deficient media were carried out by the Spectrochemistry Department of the

Institute. It was found that the Zn and Mn deficient organisms contained approximately $\frac{1}{3}$ and $\frac{1}{4}$, respectively, of the amount of these metals present in an equivalent weight of organisms grown on complete medium.

Table 2. *Effect of deficiency of iron, manganese and zinc on viable counts of* Nocardia opaca

N. opaca grown in 200 ml. complete medium, or Fe-, or Mn-, or Zn-deficient medium containing 2% (w/v) glucose at 25°. Samples removed after 5 and 7 days, and viable counts performed.

	Time of harvesting	
Medium	5 days	7 days
	Viable count × 10^{-6}/ml.	
Complete	2638	1433
−Fe	19	0·9
−Zn	710	382
−Mn	82	5·0

Effect of deficiency of trace elements on the morphology of Nocardia opaca

The absence of Fe from the culture medium produced the least effect on morphology as seen with the optical microscope, the only noticeable change being that the organisms tended to fragment somewhat sooner than organisms from complete medium. The electron micrographs of Fe-deficient organisms, however, showed that they were generally not so opaque to the electron beam and the presence of electron-dense granules could be clearly seen, often in the polar positions (Pl. 1, fig. 2). These granules have already been reported in *Nocardia opaca* (Webley, 1954). The most noticeable effect on the morphology of the organism was obtained in Mn-deficient cultures. Manganese deficiency usually resulted in a more pronounced filamentous growth of the organism, with subsequent failure to fragment into short elements (Pl. 1, figs. 3–5). On further incubation bulbous swellings frequently appeared at the end, and sometimes also along the length of filaments (Pl. 1, figs. 4–6). As the culture became older the bulbous structures tended to rupture. With Zn deficiency the filamentous growth and swellings, although sometimes present (Pl. 1, fig. 7) were usually not so marked. The bulbous swellings in Mn deficiency were opaque to the electron beam (Pl. 1, fig. 6), and stained deeply with crystal violet (Pl. 1, fig. 4). They also showed evidence of cell walls when stained with tannic acid-crystal violet (Pl. 1, fig. 5). This abnormal morphology was prevented by the addition of as little as 4–10 μg. Mn/l. (i.e. 0·004–0·01 p.p.m.) to Mn-deficient medium, but it was necessary to add about ten times this amount of Zn (0·04–0·1 p.p.m.) to correct a zinc-deficient medium. The morphological effects due to Mn deficiency were observed with all the substrates tested (glucose, sucrose, glycerol, gluconate, phenylacetate). The effects were usually seen after incubation for 5 days. With shaken cultures a similar picture was obtained, the bulbous swellings also developing under these conditions. When the organisms were harvested, washed and resuspended in double-distilled water the abnormal structures did not rupture.

DISCUSSION

The most interesting feature of the present work is the striking morphological effects obtained with *Nocardia opaca* when grown in Zn- and Mn-deficient media (particularly the latter). There appears to be no previous record of similar morphological changes in the actinomycetes. Winder & Denneny (1959) reported that the cells of *Mycobacterium smegmatis*, when grown on a modified Proskauer & Beck medium partially freed from trace elements, were elongated 'and even filament like'. The addition of 2 μg./ml. iron + 0·4 μg./ml. zinc gave the normal morphology. These workers did not test these metals singly and did not detect a response to manganese. Heim & Lechevalier (1956) found that iron was the only element that permitted a substantial increase in mycelial weight, when added singly, in the growth of eight different strains of Streptomyces; a mixture of Fe + Zn, however, gave a striking increase in mycelial weight. These workers were uncertain whether manganese was needed and did not examine the morphology of the organism. These differences in related organisms might be due either to the degree of deficiency obtained in the media or to differences in the requirements of the different organisms. The medium used here is simpler in composition than that used by Heim & Lechevalier and therefore might be somewhat more easily depleted of trace metals.

I am indebted to Mr W. Hodgkiss of the Torry Research Station, Aberdeen, for taking the electronmicrographs, to Mrs I. M. Johnston of this Institute for the spectrochemical analyses and to Miss I. Taylor and Mr M. Davidson for technical assistance.

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EXPLANATION OF PLATE

Fig. 1. *Nocardia opaca*, grown for 7 days on complete medium containing glucose. Static culture. Electron micrograph. ×14,500.

Fig. 2. *N. opaca*, grown for 7 days on Fe-deficient medium containing glucose. Static culture. Electron micrograph. ×14,500.

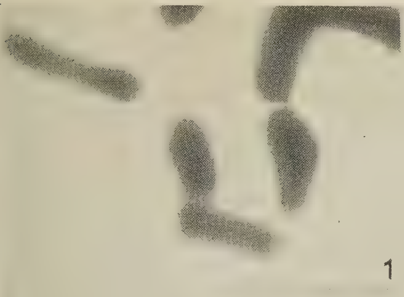
Fig. 3. *N. opaca*, grown for 3 days on Mn-deficient medium containing sucrose. Static culture. Crystal violet. ×2640.

Fig. 4. *N. opaca*, grown for 5 days on Mn-deficient medium containing sucrose. Static culture. Crystal violet. ×2640.

Fig. 5. *N. opaca*, grown for 7 days on Mn-deficient medium containing sucrose. Static culture. Cell wall stain. ×2640.

Fig. 6. *N. opaca*, grown for 10 days on Mn-deficient medium containing glucose. Shaken culture. Electron micrograph. ×14,500.

Fig. 7. *N. opaca*, grown for 7 days on Zn-deficient medium containing gluconate. Static culture. Crystal violet. ×2640.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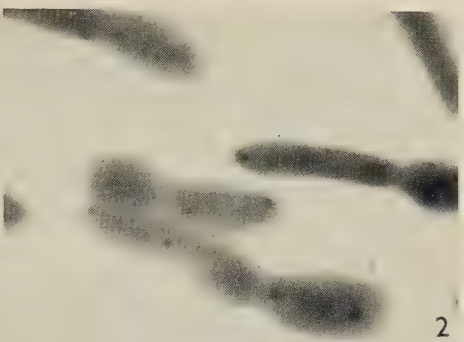
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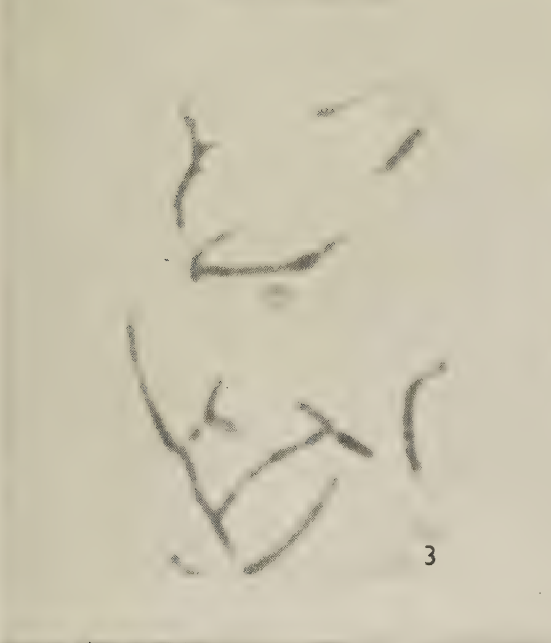
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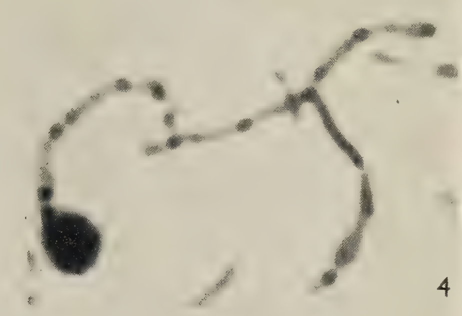
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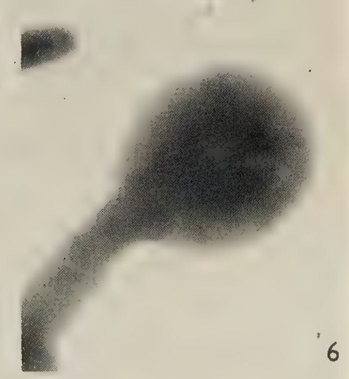
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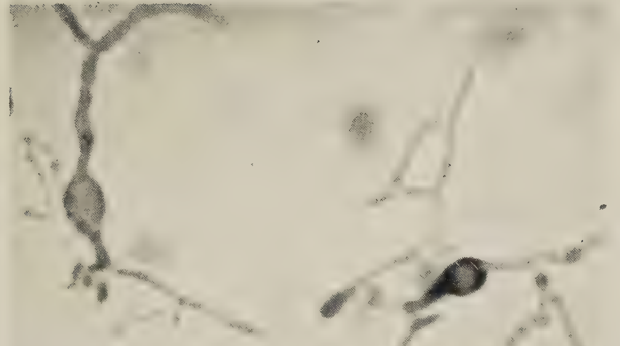
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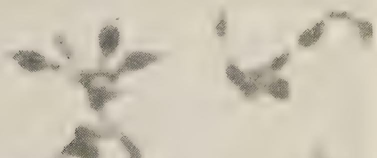
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Carotenoid Distribution in Certain Naturally Occurring Algae and in some Artificially Induced Mutants of *Chlorella pyrenoidosa*

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SUMMARY

The carotenoids of pure cultures of two members of the Class Chrysophyceae were examined; the main pigments in *Ochromonas danica* and *Prymnesium parvum* are fucoxanthin and β-carotene. The main carotenoids of *Cyanidium caldarium* are β-carotene and zeaxanthin; these observations support the view that this alga represents a transitional form between the cryptomonads and the green algae. The distribution of polyenes and carotenoids in a number of ultra-violet induced mutants of *Chlorella pyrenoidosa* is described.

INTRODUCTION

The general nature of the carotenoid pigments in many classes of algae is now reasonably well established (see Goodwin, 1958*a*; 1959*a*, *b*; 1960). However, little is known of the carotenoids in the class Chrysophyceae, the only report available being that of Heilbron (1942) in which he reported β-carotene, lutein (3,3-dihydroxy-α-carotene) and fucoxanthin (structure unknown) in a mixed culture of *Apistonema carteri*, *Thallochrysis litoralis* and *Gloeschrysis maritima*. The reasons for this dearth of information have been: (*a*) the difficulty in isolating pure strains of Chrysophyceae; (*b*) the difficulty in devising culture media on which pure strains could be cultivated in bulk in the laboratory. These difficulties have now been overcome and the first part of this paper is concerned with the nature of the carotenoids present in pure strains of *Ochromonas danica* and *Prymnesium parvum*.

The second part of the paper is devoted to a study of the carotenoids in the unique alga *Cyanidium caldarium*; taxonomically this organism can be considered to be a green alga (Chlorophyceae), although it is known to produce phycocyanins and only chlorophyll *a* (M. B. Allen, unpublished observations), which would suggest a close relationship with the Cyanophyceae (blue-green algae). However, as will be seen, its carotenoids are characteristic of neither the Chlorophyceae nor the Cyanophyceae.

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The final section of the paper deals with the carotenoid distribution in some ultraviolet induced mutants of *Chlorella pyrenoidosa*. Similar investigations on X-ray induced mutants of *C. vulgaris* have been carried out by Claes (1954).

Parallel studies on the distribution of chlorophylls in these algae will be reported elsewhere.

METHODS

Organisms and methods of cultivation

Cultures of *Ochromonas danica* and *Prymnesium parvum* were obtained from Dr J. J. A. McLaughlin. *O. danica* was grown in the defined medium shown in Table 1. *P. parvum* was grown in medium 'DC' of Provasoli, McLaughlin & Droop (1957). Both organisms were grown at 18–20° under approximately 300 f.c. of 'cool white' fluorescent light.

Table 1. *Defined medium for* Ochromonas danica

With either 0·5 % glucose added or 5 % CO_2 in air bubbled through the medium. The inorganic micronutrients are based on the A 4 and B 7 solutions of Arnon (1938).

Macronutrients		Micronutrients	
	(M)		(mg./l.)
$MgSO_4$	0·001	Thiamin	10
KNO_3	0·005	Biotin	2
$(NH_4)_2SO_4$	0·005	Fe	4
$CaCl_2$	0·0005	Mn	0·5
KH_2PO_4	0·002	B	0·5
		Zn	0·05
		Cu	0·02
		Mo	0·01
		V	0·01

Mutants of *Chlorella pyrenoidosa* (Emerson strain; culture no. C–l. 1.10 in the Kaiser Foundation Research Institute culture collection) were obtained by ultraviolet irradiation with a Hanovia UV lamp. On plating the irradiated cultures, a large number of pigment mutants were found. With one exception (G 34) those investigated in the present work were originally of interest because their chlorophyll synthesis is disturbed so that they produce only chlorophyll *a* (Allen, 1958). The mutants were grown in a medium containing: $MgSO_4$, 0·001 M; KNO_3, 0·01 M; NaCl, 0·004 M; $CaCl_2$, 0·0005 M; K_2HPO_4, 0·002 M; glucose 0·5 %; inorganic micronutrients shown in Table 1. Mutants G-41 and G-44 were grown in dim light (100 f.c.); mutant G-34 was grown in the dark, as it is photosensitive.

Extraction, separation and identification of pigments

The harvested cultures were freeze-dried, sealed under nitrogen and sent air mail from Berkeley to Liverpool. It was shown in a previous investigation that this transportation has no ill effects on the carotenoids in dried algal cells (Goodwin & Gross, 1958). The dried organisms were weighed and then ground with acid-washed silver sand under acetone in a small mortar. The acetone extract was filtered through a sintered glass crucible (P × 4) and the process continued until the final acetone extract was colourless. The combined acetone extracts were then transferred to a separating funnel and an equal volume of diethyl ether (freshly distilled over reduced iron to remove peroxides) added; this was followed by adding water slowly until two layers formed. All the pigment was present in the upper ethereal layer, which was washed several times with successive small amounts of water until free from acetone. The ether was removed *in vacuo* and the residue was either chromatographed directly or saponified before chromatography. Except in cases where fucoxanthin was present, the extracts were saponified before chromatography; this removed chlorophylls and lipids which might interfere with the development of chromatograms. Fucoxanthin is the only known carotenoid which is destroyed by alkali, so when this pigment was present the saponification step was omitted.

Saponification of the extracts was carried out by the standard method in use in this laboratory (Goodwin, 1955). The extracts were generally dissolved directly in 2 ml. light petroleum (b.p. 40–60°) ready for chromatography. When the extracts contained large amounts of fucoxanthin or other polyhydroxylated carotenoids (which are insoluble in light petroleum) they were first dissolved in 2–5 drops of ether and then diluted to an appropriate volume with light petroleum. A number of different adsorbents have been used to separate the pigments. Icing sugar (confectioner's sugar, Tate and Lyle Ltd.) was ground in a mortar and activated by heating in an oven at 80° for 6 hr. and then cooling in a desiccator; this adsorbent was used to separate xanthophylls. Alumina was used either activated (Grade 'O', Peter Spence and Sons Ltd., Widnes, Lancashire) or after deactivation according to the method of Goodwin & Srisukh (1949). Activated alumina was used for separating the partly saturated polyene hydrocarbons phytoene and phytofluene from the more unsaturated compounds such as ζ-carotene, neurosporene and β-carotene. Both phytoene and phytofluene are colourless; the latter was located on the column by its characteristic green fluorescence under ultraviolet irradiation. There is, however, no way easily to detect phytoene and it was obtained by collecting all the eluate running before phytofluene in 10 ml. fractions and bulking those fractions which exhibited the characteristic absorption peak of phytoene at 286 mμ (Goodwin, 1952*a*, 1955). Deactivated alumina was used for the rapid separation of β-carotene from oxygen-containing carotenoids.

Zinc carbonate + celite (4:1, w/w) was used extensively for the separation and purification of xanthophylls, and magnesium oxide + celite (4:1, w/w) for the purification of β-carotene. Both were activated by heating to 180°, and stored in the same way as icing sugar.

Alumina columns were prepared by using a slurry, whereas all other adsorbents were packed dry (Goodwin, 1955). The chromatograms were developed by the method of gradient elution. The solvent used in all cases was light petroleum (40–60°) containing progressively increasing amounts of acetone; the apparatus used was very similar to that described by Lederer & Lederer (1957); the original solvent was light petroleum (b.p. 40–60°) and the final solvent light petroleum containing 10% (v/v) acetone. Xanthophylls are separated much more clearly, with little tailing, when gradient elution is used.

The individual pigments were identified (*a*) by their position on the adsorption column relative to other known pigments, (*b*) by the shape of their absorption curves and the position of the absorption maxima in various solvents, (*c*) by mixed chromatography with an authentic specimen (Goodwin, 1955). The quantitative determination of the various pigment fractions was carried out as described previously (Goodwin, 1955) using the $E^{1\%}_{1\,\mathrm{cm.}}$ values for each pigment quoted by Goodwin (1955). When the $E^{1\%}_{1\,\mathrm{cm.}}$ value of a pigment was unknown, it was assumed to be the same as that of β-carotene, i.e. 2500.

Partition tests were also applied to the isolated pigments. To carry out these tests a solution of the pigment in light petroleum was shaken with an equal volume of 90 or 95% (v/v) methanol in water in a small test tube (ignition tube). In both tests hydrocarbons remain in the upper layer (epiphase). When 95% methanol in water was the lower phase then all xanthophylls are hypophasic; with 90% methanol in water the xanthophylls containing one oxygen function remain with the hydrocarbons in the epiphase whilst the remaining xanthophylls are hypophasic (Goodwin, 1955).

The pigment fractions were examined spectrophotometrically by using either a Unicam SP. 500 or SP. 600 instrument.

RESULTS

Carotenoids in Ochromonas danica *and* Prymnesium parvum (*Chrysophyceae*)

Ochromonas danica. Preliminary tests indicated the presence of fucoxanthin, so the step involving saponification had to be omitted. On chromatography on icing sugar, three main carotenoids were obtained (Table 2; the chlorophyll bands were ignored). Fraction A, which passed straight through the column with only light petroleum as developer, was purified on deactivated alumina and its identity with β-carotene was confirmed by co-chromatography with an authentic crystalline specimen (synthetic); no separation was obtained. The general properties of fraction B, which was developed with light petroleum containing 3% (v/v) acetone, including its destruction by alkali, suggested that it was fucoxanthin. This was confirmed by comparing it with an authentic specimen isolated from *Laminaria saccharina* (Phaeophyceae). Both pigments had the same absorption spectrum and could not be separated on co-chromatography on either icing sugar or a $ZnCO_3$ + celite mixture. Fraction C which was firmly adsorbed on the column and was present only in small amounts was not identified. Quantitative determinations of the pigment fractions (Table 2) showed that fucoxanthin was the predominating pigment and that the total amount of pigment present was more than that recorded for various Phaeophyceae (see Goodwin, 1952*b*).

Prymnesium parvum. The pigment distribution in this alga was very similar to that observed in *Ochromonas danica*, but minor differences did exist. Fraction A

Table 2. *The carotenoids present in* Ochromonas danica

Pigment extract chromatographed on icing sugar; fractions listed in order of increasing adsorptive power.

Fraction	Description	Absorption maxima (mμ) in light petroleum	Identification	Concn. (mg./g. dry wt.)	% of total pigment
A	Yellow	448, 475	β-Carotene	0·725	17·0
B	Orange	448, 475	Fucoxanthin	3·160	73·8
C	Deep yellow	445	Unknown	0·395	9·2
			Total	4·28	

(Table 3) on purification on deactivated alumina separated into two zones; the lower yellow zone was shown to be β-carotene. The other zone was not unequivocally identified; it exhibited absorption maxima (light petroleum) at 424, 445 and 470 mμ and was probably a *cis* isomer of β-carotene. Fraction B showed properties very similar to that of zeaxanthin except that its absorption maxima in light petroleum were slightly higher; however, on co-chromatography with an authentic specimen of zeaxanthin prepared from maize the two fractions could be separated. Pigment B was therefore in all probability diatoxanthin, a pigment very similar to zeaxanthin found in diatoms by Strain, Manning & Hardin (1944). Diatoxanthin according to Strain *et al.* has absorption maxima at slightly higher wavelengths than has zeaxanthin and, as just stated, this was also true for pigment B. A comparison of the absorption maxima of the three pigments is given in Table 4. Pigments C and D were present only in trace amounts and could not be unequivocally identified with any known pigment. Fraction E was proved to be fucoxanthin using the same criteria as those described for *O. danica*, and fraction F appeared to be the same pigment as fraction C (Table 3) from *O. danica*. The total pigment concentration was over twice as great as in *O. danica*, but fucoxanthin still represented about 75% of the total pigments present.

Table 3. *The carotenoids present in* Prymnesium parvum

Pigment extract chromatographed on icing sugar; fractions listed in order of increasing adsorptive power.

Fraction	Description	Absorption maxima (mμ) light petroleum	Identification	Concn. (mg./g. dry wt.)	% of total pigment
A	Yellow	—	β-carotene + a *cis*-isomer	0·70	7·4
B	Orange-yellow	450, 480	Diatoxanthin	0·65	6·1
C	Orange-yellow	420, 445–7	Unknown	0·28	2·7
D	Yellow	425, 450	Unknown	0·52	4·8
E	Orange	448, 475	Fucoxanthin	7·91	74·3
F	Yellow	445	Unknown—same as fraction C (Table 1)	0·51	4·7
			Total	10·57	

Table 4. *A comparison of the absorption maxima* (mμ) *of zeaxanthin, diatoxanthin and pigment B* (*Table* 2)

	Solvent. Light petroleum (b.p. 40–60)	Ethanol
Zeaxanthin (ex maize; present work)	448, 476–478	450, 478
Diatoxanthin (Strain *et al.* 1944)	—	453, 478
Pigment B	450, 480	452, 481

Carotenoids in Cyanidium caldarium

The carotenoids present in *Cyanidium caldarium* were found to be β-carotene, zeaxanthin and lutein, with β-carotene predominating (Table 5). β-Carotene was identified in the usual way, whilst the identity of zeaxanthin was confirmed by co-chromatography with authentic zeaxanthin on two adsorbents: icing sugar and $ZnCO_3$+celite (4:1); no separation was obtained in either case. Fraction C was similarly identified by comparison with an authentic specimen of lutein isolated from grass. The total pigment concentration is very much less than in the two members of Chrysophyceae examined.

Table 5. *The carotenoids present in* Cyanidium caldarium

Pigment extract chromatographed on icing sugar; fractions listed in order of increasing adsorptive power.

Fraction	Description	Absorption maxima (mμ) light petroleum	Identification	Conc. (mg./g. dry wt.)	% of total pigment
A	Yellow	448, 473	β-Carotene	0·220	54·4
B	Orange-yellow	448, 475	Zeaxanthin	0·129	31·8
C	Yellow	443, 472	Lutein	0·057	13·8
			Total	0·406	

Carotenoids in mutants of Chlorella pyrenoidosa

Mutant G. 34. The saponified extracts of this mutant were colourless and thus no carotenoids were present. However, chromatography on activated alumina demonstrated the presence of a colourless polyene, very similar to phytoene. As this compound is neither coloured nor fluorescent, the chromatogram was developed with light petroleum containing 0·2% (v/v) acetone and the eluate collected every 10 ml. A material with an absorption spectrum identical in shape and position of phytoene was obtained. Its distribution in the fractions collected was determined and in order to confirm the identity of this polyene with phytoene, an authentic specimen of phytoene was prepared from carrots, mixed with an equal amount of the mutant polyene and chromatographed as just described. The distribution curves for authentic phytoene and for the mixture were identical with that for the unknown polyene. This clearly demonstrated the identity of the two substances. The concentration of phytoene in the mutant was 1·71 mg./g. dry wt. which is of the same order as that for the total carotenoids in the wild strain of *C. pyrenoidosa* from which the mutant was derived (see Table 9).

Mutant G. 44. When the carotenoids of this mutant were chromatographed on icing sugar it was found that the greater part of the fraction passed rapidly through the column; that is, the main components were hydrocarbon in nature. Chromatography of this fraction on activated alumina yielded the fractions listed in Table 6. Fraction A 1 was identified as phytofluene by the position and shape of its absorption maxima, by its green fluorescence under ultraviolet irradiation and by its failure to separate from an authentic specimen of phytofluene (from carrots) on co-chromatography. Fraction A 2 was further purified on MgO+celite (3:1, w/w) and the major fraction had the same absorption spectrum and adsorptive power as ζ-carotene (Nash & Zscheile, 1945), but complete conformation could not be obtained because no authentic specimen of ζ-carotene was available. The minor component of fraction A 2 was shown to be β-carotene in the usual way. Fractions A 3 and A 4 contained traces of pigments with the same absorption spectrum as ζ-carotene but which were more strongly adsorbed. They were not examined further but they may have been monohydroxy derivatives of ζ-carotene. It is interesting that although phytofluene is present in the mutant, phytoene is absent.

The xanthophyll fraction (B) was removed from the icing sugar and rechromatographed on $ZnCO_3$ + celite (4:1, w/w) using gradient elution. Six fractions were obtained, the absorption spectra of which are recorded in Table 7. As the xanthophylls represented only about 15 % of the total pigments and as each fraction was present in approximately equal amounts there was not sufficient material for detailed examination. However, it is highly likely that B 2 is lutein and that B 3 is a di- or poly-hydroxy derivative of ζ-carotene or of a polyene with a chromophoric system very similar to that of ζ-carotene. This appears to be the first time that such pigments have been reported. B 1, which is only present in traces, may be a hydroxylated derivative of ζ-carotene or a *cis* isomer of the corresponding ζ-carotene derivative (B 3). B 4 might be auroxanthin or a hydroxylated ζ-carotene, but it was not investigated further, and B 5, which also could not be immediately identified, might be a degradation product.

Table 6. *The hydrocarbon polyenes present in mutant* G. 44 *of* Chlorella pyrenoidosa

Pigments separated on MgO + celite mixture; fractions listed in order of increasing adsorptive power.

Fraction	Description	Absorption maxima (mμ) light petroleum	Identification	Concn. (mg./dry wt.)	% of total hydrocarbon polyenes
A 1	Colourless; intense green fluorescence	331, 348, 367	Phytofluene	0·455	16·6
A 2	Yellow	378, 399, 424	ζ-Carotene	2·090	76·0
A 3	Pink	376, 399, 423	Unknown	0·066	2·4
A 4	Yellow	377, 398, 424	Unknown	0·139	5·0
				2·740	

Table 7. *The xanthophylls present in mutant* G. 44 *of* Chlorella pyrenoidosa

Pigments separated on $ZnCO_3$ + celite (4:1, w/w) by means of gradient elution; fractions listed in order of increasing adsorptive power.

Fraction	Description	Absorption maxima (mμ) light petroleum	Identification	Concn. (mg./g. dry wt.)	% of total carotenoids
B 1	Yellow	374, 395, 418	Unknown	0·008	0·2
B 2	Orange	421, 443, 470	Lutein	0·064	2·0
B 3	Yellow	374, 397, 423	Hydroxy derivative of ζ-carotene or auroxanthin	0·180	5·5
B 4	Yellow	397, 415, 438	Unknown	0·114	3·3
B 5	Yellow	432, 460	Unknown	0·077	2·3
			Total	0·443	13·3

Mutant G. 41. The pigments detected in this mutant are summarized in Table 8. The general distribution was very similar to that observed in the wild strain and the individual pigments were identified in the usual manner. There are, however, two minor differences: (*a*) α-carotene is not present in the mutant; (*b*) a mixture which could not be successfully separated and identified was different from the unknown fractions in the wild strains.

Table 8. *The carotenoids present in mutant* G. 41 *of* Chlorella pyrenoidosa

Xanthophylls separated on icing sugar; hydrocarbons on activated alumina; in both cases pigments were eluted with light petroleum containing increasing concentrations of acetone; pigments in order of increasing adsorptive power.

Fraction	Description	Absorption maxima (mμ) light petroleum	Identification	Concn. (mg./g. dry wt.)	% of total carotenoids
A	Yellow	425, 448, 473	β-Carotene	0·513	20·3
B 1	Orange	443, 472	Lutein	0·926	36·7
B 2	Yellow	Mixture	Unknown	0·690	26·4
B 3	Yellow	418, 440, 468	Violaxanthin	0·354	14·0
B 4	Yellow	415, 437, 468	Neoxanthin	0·039	1·6
			Total	2·422	

Carotenoids present in Chlorella pyrenoidosa *wild-type*

In order to compare the pigments in the mutants with those of the parent strain the pigments present in *Chlorella pyrenoidosa* (wild-type) were examined; the results are summarized in Table 9. It will be seen that the main pigments are very similar to those reported in other green algae (Goodwin, 1952*b*, 1955). It is interesting, however, that α-carotene is present, although it is absent from most strains of *C. vulgaris* (Goodwin, 1955).

Table 9. *The carotenoids present in* Chlorella pyrenoidosa *wild-type*

Separation as described in Table 8 for mutant G 41 of *C. pyrenoidosa.*

Fraction	Description	Absorption maxima (mμ) light petroleum	Identification	Concn. (mg./g. dry wt.)	% of total carotenoids
A 1	Yellow	420, 445, 472	α-Carotene	0·09	3·6
A 2	Orange-yellow	425, 448, 473	β-Carotene	0·33	13·5
B 1	Yellow	442, 470	Lutein	1·11	54·8
B 2	Yellow	415, 438, 466	Violaxanthin	0·22	9·0
B 3	Pale yellow	399, 422, 443	Unknown	0·10	4·2
B 4	Deep yellow	415, 440, 467	Unknown	0·15	6·1
B 5	Yellow	417, 435, 463	Neoxanthin	0·21	8·8
			Total	2·21	

DISCUSSION

The Chrysophyceae. The present work on pure cultures of *Ochromonas danica* and *Prymnesium parvum* confirms the earlier work of Heilbron (1942) who found β-carotene and fucoxanthin in a mixed culture of *Apistonema carteri*, *Thallochrysis litoralis* and *Gloeschrysis maritima*; it also demonstrates that fucoxanthin is by far the predominant pigment, representing some 75% of the total carotenoids present.

Thus qualitatively the carotenoid distribution in these two examples of the class Chrysophyceae resembles very closely that found in the Phaeophyceae. The total amounts of pigment produced by the two algae vary considerably (Tables 2 and 3), but in any case the concentration in these two unicellular Chrysophyceae is much greater than that in the colonial Phaeophyceae for which values ranging from 0·36 to 1·9 mg./g. dry wt. have been recorded (Seybold & Egle, 1938). Indeed *P. parvum* (10·57 mg./g. dry wt.) now becomes, to the best of our knowledge, the richest known algal source of carotenoids, the previous highest value being *c.* 7·0 mg./g. dry wt. recorded for *Euglena gracilis* v. *bacillaris* (Goodwin & Jamikorn, 1954). The diatoms represent the other main class of algae which synthesize large amounts of fucoxanthin, and a relationship between the Chrysophyceae and this group is further indicated by the existence in *P. parvum* of diatoxanthin, a pigment closely related to zeaxanthin and previously considered unique to diatoms (Goodwin, 1952*b*).

Cyanidium caldarium

Cyanidium caldarium is perhaps the most interesting of the recently examined algae. Taxonomically it can be considered a green alga, although its claim to be classed as a blue-green alga would appear strong on purely biochemical grounds when it is realized that it synthesizes phycocyanins and only chlorophyll *a* (both characteristics of blue-green algae). The component carotenoids are, however, not characteristic of either the Cyanophyceae or the Chlorophyceae. They consist of a relatively large proportion of β-carotene (55 % of the total pigments) with zeaxanthin as the main xanthophyll. The high proportion of β-carotene is reminiscent of the blue-green algae (Goodwin, 1958*b*), although *Chlamydomonas reinhardii* is also reported to contain comparative large amounts of β-carotene (Sager & Zalokar 1958). The only other alga which is known to produce zeaxanthin or a closely related pigment, in comparatively large amounts, is *Cryptomonas ovata*, the first member of the Cryptophyceae to be examined in pure culture (Haxo & Fork, 1959). The sporadic reports of zeaxanthin in some Phaeophyceae have been shown by Liaaen & Sörensen (1956) to be due to the conversion of violaxanthin into zeaxanthin, brought about by drying the algae without all necessary precautions. The carotenoid distribution in *C. caldarium* is not incompatible with the view that it is a transitional form between the cryptomonads and the green algae, because it is possible that the cryptomonads represent a primitive flagellate groups from which the Chlorophyceae have developed. Quantitatively the amount of carotenoids synthesized is rather low (see Goodwin, 1952*b*).

Mutant strains of Chlorella pyrenoidosa

It is interesting to compare the pigments produced by various mutants of *Chlorella pyrenoidosa* with those in the mutant strains of *C. vulgaris* previously described by Claes (1954) (summarized in Table 10). In the first place *C. pyrenoidosa* mutants G. 34 and *C. vulgaris* mutant 5/871 are very similar in that they produce only phytoene; furthermore, in G. 34 the amounts produced are approximately the same as the total amount of carotenoid synthesized by the parent strains. Both are light-sensitive in the presence of oxygen. *C. pyrenoidosa* G. 44 and *C. vulgaris* 5/515 and 9a all accumulate phytofluene and ζ-carotene, but unlike Claes's mutants, G. 44 does not synthesize phytoene. Furthermore, in G. 44 xanthophyll production was

Table 10. *Polyene distribution in mutants of* Chlorella pyrenoidosa *and* C. vulgaris

+ = present; − = absent.

	C. pyrenoidosa				*C. vulgaris*				
	Wild-type*	G 34	G 41	G 44†	Wild-type	5/871	5/515	5/520‡	9*a*§
Phytoene	−	+	−	−	−	+	+	+	+
Phytofluene	−	−	−	+	−	−	+	+	+
β-Carotene	+	−	+	−	+	−	−	−	−
ζ-Carotene	−	−	−	+	−	−	+	+	+
Protetrahydrolycopene	−	−	−	−	−	−	−	+	−
Prolycopene	−	−	−	−	−	−	−	+	−
Lutein	+	−	+	+	+	−	−	−	+
Violaxanthin	+	−	+	−	+	−	−	−	+
Neoxanthin	+	−	+	−	+	−	−	−	+

* Also synthesizes α-carotene.
† Also synthesizes a hydroxylated ζ-carotene.
‡ These pigments are formed in the dark; normal plastid pigments are produced in the light.
§ An unidentified carotene is also present.

not completely inhibited (compare mutant 5/515), but it was decreased in amount and qualitatively altered (compare mutant 9a); only about 20% of the total pigments were xanthophylls and they appeared to be mainly either hydroxylated derivatives of ζ-carotene or compounds closely related to auroxanthin. It was not possible to decide between these alternatives. Our mutant G. 41 is interesting in that its ability to synthesize carotenoids has undergone little qualitative or quantitative change; its chlorophyll-synthesizing system has, however, been profoundly affected in that it does not synthesize chlorophyll *b* (Allen, 1958).

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The Synthesis of Serine by *Leuconostoc mesenteroides* P60

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SUMMARY

Though CO_2 is essential for the growth of *Leuconostoc mesenteroides* strain P60 in the absence of serine, $^{14}CO_2$ is not incorporated into serine by the growing organism. The synthesis of serine was further studied by using suspensions of a variant strain of *L. mesenteroides* P60 which formed serine more actively than the parent strain. Glycine, formate, glucose, *p*-aminobenzoic acid and pyridoxal were required for the synthesis of serine by these suspensions. There was an additional requirement for CO_2 only when the organisms had been incubated previously in phosphate-buffered glucose. Leucovorin satisfied the requirement for both CO_2 and *p*-aminobenzoic acid.

INTRODUCTION

Evidence for the biosynthesis of serine from glycine by *Leuconostoc mesenteroides* strain P60, and the part played therein by vitamin B_6 and growth factors of the folic acid group, has largely been derived from nutritional experiments (Steele, Sauberlich, Reynolds & Baumann, 1949; Wold & Sirny, 1953; Lascelles, Cross & Woods, 1954; Sauberlich, 1954). In order that this organism shall grow in the absence of serine not only must the concentration of glycine and *p*-aminobenzoic acid (*p*-AB) be increased well above those found to be optimal for growth in the presence of serine, but pyridoxal must also be added to the medium, and the gas phase enriched with CO_2. Lyman *et al.* (1947) reported a similar replacement of amino acids for the growth of *Streptococcus faecalis* strain R, for which CO_2 and vitamin B_6 substituted for aspartic acid, and for the growth of *Lactobacillus arabinosus*, where CO_2 and vitamin B_6 substituted for arginine, phenylalanine and tyrosine. Carbon dioxide also increases the growth of *Streptobacterium plantarum* strain 10S in a medium containing homocysteine in place of methionine, whereas maximum growth is achieved in N_2 in the presence of methionine (Cross, 1954). This replacement of methionine by homocysteine + CO_2 parallels more closely the replacement of serine by CO_2 + glycine, in that, in both cases, CO_2 is apparently substituting for a 'labile methyl group'.

It has already been reported briefly that $^{14}CO_2$ is not incorporated into serine by growing cultures of *Leuconostoc mesenteroides* P60 (Cross, 1953; Lascelles *et al.* 1954); these experiments will be fully described here. The situation is analogous to that found with *Streptobacterium plantarum* 10S which does not incorporate $^{14}CO_2$ into the methyl group of the synthesized methionine when growing in the absence of the amino acid.

The main object of the present work was the further study of the role of CO_2 in serine synthesis under the more controlled conditions possible with suspensions of organisms; the initial design of experiments was guided largely by the observations of Lascelles & Woods (1954) with suspensions of *Streptococcus faecalis* R which form serine in a medium containing glycine, formate, glucose, pyridoxal and pteroylglutamic acid.

METHODS

The variant strain of Leuconostoc mesenteroides *P* 60. This was derived from *L. mesenteroides* P 60 (*Streptococcus equinus* P 60) (ATCC 7881), which was maintained as described by Lascelles *et al.* (1954). After 15 subcultures in medium *L* supplemented with 10^{-2}M-glycine, 10^{-6}M-*p*-aminobenzoic acid and 2×10^{-7}M-pyridoxal in an atmosphere of 5% (v/v) CO_2 + 95% (v/v) N_2 at 37° the culture showed an enhanced ability to grow in the absence of added serine.

The variant strain was maintained in liquid medium *L* supplemented as above, subcultured every week and stored at 4°.

Growth experiments with strains of Leuconostoc mesenteroides *P* 60. Inocula for nutritional experiments to compare the two strains were derived from fresh stock cultures of the organisms: in medium B_1 (Nimmo-Smith, Lascelles & Woods, 1948) for the parent strain, and in medium *L* supplemented as already described for the variant strain. Both strains were subcultured in supplemented medium *L* and incubated for 24 hr. at 37° in 5% (v/v) CO_2 + 95% (v/v) N_2. The cultures were diluted 1/50 for use as an inoculum. In other respects the basal medium and procedure were as described by Lascelles *et al.* (1954). Each tube of test medium was inoculated with 0·1 ml. of the inoculum. The procedure in experiments involving the parent strain only was exactly as described by Lascelles *et al.* (1954).

Preparation of suspensions of the variant strain of Leuconostoc mesenteroides *P* 60

Medium. Organisms deficient in both *p*-aminobenzoic acid and vitamin B_6 were obtained by growth on medium (*C*) based on that described by Lascelles & Woods (1954) for the growth of *Leuconostoc citrovorum.* It contained in each litre of final medium: acid-hydrolysed casein (vitamin free, prepared by the method of Snell & Rannefeld, 1945) equivalent to 5 g. original casein; DL-cysteine, 100 mg.; DL-alanine, 870 mg.; DL-tryptophan, 100 mg.; glucose, 10 g.; acetic acid (glacial), 4·0 ml.; $(NH_4)_2SO_4$, 8 g.; KH_2PO_4, 2 g.; $MgSO_4.7H_2O$, 40 mg.; $MnSO_4.4H_2O$, NaCl and $FeSO_4.7H_2O$, 20 mg. each; adenine, guanine and uracil, 10 mg. each; nicotinic acid, 1 mg.; riboflavin, 500 μg.; thiamin monohydrochloride and calcium pantothenate, each 600 μg.; biotin, 1 μg. *p*-Aminobenzoic acid was added at a final concentration 5×10^{-10}M; the pH value was adjusted to 6·9.

p-Aminobenzoic acid could be omitted from the medium when thymidine was added (Lascelles *et al.* 1954). However, organisms deficient in *p*-aminobenzoic acid were obtained when suboptimal concentrations of this vitamin were present, and this procedure was preferred in order to spare stocks of thymidine.

Growth. Medium *C* (200 ml. quantities in 300 ml. conical flasks) was autoclaved for 7 min. at 10 lb./sq.in. and warmed at 37° before inoculation with 0·1 ml. (about 2×10^8 organisms) from a 24 hr. culture on the supplemented medium *L* at 37° in 5% (v/v) CO_2 + 95% (v/v) N_2. The flasks were incubated for 10–11 hr. at 37° in air. The culture then contained about 0·05 mg. dry wt. organisms/ml., less than 10% of the maximum density attainable in the medium supplemented with optimal concentrations of pyridoxal and *p*-aminobenzoic acid. The organisms were centrifuged down, and washed in the culture volume of 2×10^{-2}M-phosphate buffer (pH 6·9).

General procedure for the study of serine synthesis

Reaction mixture. The washed organisms were suspended at a concentration equivalent to 0·2–0·4 mg. dry wt./ml. in 2·5 ml. quantities (in 25 ml. Pyrex conical flasks) of the solution of substrates. The complete solution (D) normally contained for synthesis of serine: glycine, 2×10^{-2}M; glucose 10^{-2}M; sodium formate, 10^{-2}M; pyridoxal, 10^{-6}M; *p*-aminobenzoic acid, 10^{-5}M; phosphate buffer, pH 6·9, 10^{-1}M (prepared by mixing 10^{-1}M solutions of Na_2HPO_4 and KH_2PO_4). Modifications in particular experiments are noted in the text. Incubation was at 37° and usually for 24 hr. over which period the dry wt. of organisms decreased about 15% in typical systems. For atmospheres other than air, adapted 'Kilner' fruit-preserving jars (Nimmo-Smith *et al.* 1948) were used. 'Boiled control' flasks contained heated organisms (4 min., 100°) suspended in the complete mixture.

In some experiments the organisms were incubated in 2×10^{-2}M-glucose and 10^{-1}M-phosphate buffer (pH 6·9) before testing their synthetic ability. This was carried out with the same concentration of organisms as above in a final volume of 35 ml. for 3 hr. at 37° in air. The organisms were centrifuged down and re-suspended in solution D modified as required.

Microbiological assay of serine. The preparation of material for the assay of serine and the assay itself with *Leuconostoc mesenteroides* P 60 was carried out as described by Lascelles *et al.* (1954) and Lascelles & Woods (1954). The growth response to DL-serine and to typical samples corresponded over a wide range of concentration (Fig. 1). Results are expressed as mM-L-serine (the assay organism responds only to L-serine). There was little variation in the quantity of serine synthesized when the concentration of organisms was increased beyond the range used (equiv. 0·25–0·4 mg. dry wt. organisms/ml.) so that the results are not significantly affected by such changes. The over-all accuracy of the method was about ±10% for concentrations of L-serine in the range 0·05–1·0 mM. The procedure was checked with typical samples by assay in the absence of pyridoxal (Lascelles *et al.* 1954).

*The chromatographic identification of serine by using Dowex-*50 *resin.* Serine synthesized by suspensions of the variant strain of *Leuconostoc mesenteroides* P 60 was separated chromatographically on Dowex-50 resin (Moore & Stein, 1951). The amino acid fractions were located by estimation of amino-nitrogen with ninhydrin (Moore & Stein, 1948; Lascelles & Woods, 1954).

Growth experiments with isotopically labelled substrates. To investigate the incorporation of ^{14}C-labelled CO_2, sodium bicarbonate and sodium formate into serine by growing cultures of *Leuconostoc mesenteroides* P 60 the organism was grown in 200 ml. quantities of medium L supplemented with 10^{-2}M-glycine, 10^{-6}M-*p*-aminobenzoic acid and 2×10^{-7}M-pyridoxal (added after autoclaving) in 500 ml. flat-bottomed flasks fitted with a side-arm. The medium, with the addition of [^{14}C]-sodium formate (10^{-2}M) when required, was autoclaved 10 min. at 10 lb./sq.in. The inoculum was 0·2 ml. of an 18 hr. culture in medium B_2 derived in turn from stock B_1. Each flask was filled with N_2 to a partial pressure of about 95% atmospheric by alternate evacuation and washing with that gas. Then $^{14}CO_2$ was generated from the calculated quantity of [^{14}C]barium carbonate + excess HCl in a Rittenberg tube attached to the side arm (to give a final concentration of 2–5% (v/v) CO_2 in the flasks); or, alternatively, a solution of [^{14}C]sodium bicarbonate was introduced through a tap funnel to a final concentration of 5×10^{-3}M. Incubation was for 30–46 hr. at 37°. The apparatus was then flushed with N_2 and the organisms centrifuged down and washed with 10^{-2} N-HCl and water. After drying *in vacuo* over P_2O_5 the yield was about 80–110 mg. Control experiments were performed with and without additions to the basal medium of inactive substrates and DL-serine, and uninoculated flasks were incubated for several days. These experiments indicated that the sterile techniques adopted in using this apparatus were satisfactory.

The isolation of glycine and serine as their 2:4-*dinitrophenyl derivatives.* Organisms grown in the presence of isotopically labelled nutrients were hydrolysed with 6N-HCl at 105° in sealed tubes for 16 hr. and the HCl then removed *in vacuo.* The product was allowed to react with 1-fluoro-2:4-dinitrobenzene and the mixture of dinitrophenyl derivatives fractionated on 'Celite 545' (Johns Manville Co. Ltd., London, S.E. 11). The DNP-serine and DNP-glycine fractions were purified and their content estimated. After dilution with previously prepared DNP-L-serine and DNP-glycine the samples were recrystallized until they showed no further change of radioactivity. The procedure followed throughout was that of Campbell & Work (1952).

Radioactivity measurements. Samples were mounted at 'infinite thickness' on 1 sq.cm. polythene disks (Popják, 1950) and measurements were made with a thin mica end-window Geiger counter. Combined errors amount to about ±5%. Results are recorded in counts/min. under these conditions.

Chemicals used. The pteroylglutamic acid used was 'Folvite' (Lederle Laboratories, Pearl River, New York, U.S.A.). [^{14}C]Barium carbonate, sodium bicarbonate (in aqueous solution) and sodium formate, were supplied by the Radiochemical Centre, Amersham, Buckinghamshire, England. The source of all other materials, and the preparation and storage of stock solution, were described by Lascelles *et al.* (1954)

RESULTS

The incorporation of [^{14}C] *formate into serine by growing cultures of* Leuconostoc mesenteroides *P* 60

The relationship between the concentrations of glycine and CO_2 required by *Leuconostoc mesenteroides* P 60 for growth in the absence of serine (Lascelles *et al.* 1954) led to the suggestion that CO_2 might serve as a source of the one-carbon residue, necessary for the conversion of glycine to serine (Lascelles *et al.* 1951). Though the observations with suspensions of the variant strain of the organism (see below) appear to suggest a different role for CO_2, the most direct evidence against the possibility that CO_2 is such a one-carbon donor was that obtained by using ^{14}C-labelled CO_2.

Cultures of *Leuconostoc mesenteroides* P 60 were grown for 30–46 hr. in medium *L* supplemented with glycine, *p*-aminobenzoic acid and pyridoxal in atmospheres containing 2–5% (v/v) $^{14}CO_2$ + 95% (v/v) N_2; and in N_2 with the addition of 5×10^{-3}M-[^{14}C]sodium bicarbonate to the medium. Very little activity was found in the whole dried organisms. The dried organisms were hydrolysed with 6N-HCl and the hydrolysate fractionated by paper chromatography. No activity was detected in either serine or glycine, though incorporation of CO_2-carbon into serine would have been observed by this technique had the dilution been as great as 1 in 400. In one experiment serine and glycine were isolated as their 2:4-dinitrophenyl derivatives. These showed no activity.

On the other hand, when *Leuconostoc mesenteroides* P 60 was grown for 46 hr. in the presence of unlabelled sodium bicarbonate under the same conditions but with addition of 5×10^{-3} M-[^{14}C]sodium formate ($1{\cdot}3 \times 10^6$ counts/min./sq.cm. at infinite thickness), the DNP-L-serine isolated from the hydrolysate of the organisms was labelled ($5{\cdot}6 \times 10^4$ counts/min./sq.cm. at infinite thickness). DNP-glycine isolated from the organisms showed no significant activity. Assuming that all the activity in the serine was located in the β-carbon atom, the dilution of the exogenous formate-carbon entering the position was about 1 in 6. Such dilution was to be expected since exogenous formate does not even stimulate growth of the organism in the absence of serine.

Growth characteristics of the variant strain of Leuconostoc mesenteroides *P* 60

The requirements of the variant strain for serine, and for glycine in the absence of serine, differ from those of the parent strain (Lascelles *et al.* 1954) in that (*a*) some growth occurred in the absence of serine in the presence of only 5×10^{-4} M-glycine, under which conditions there was stimulation by CO_2; (*b*) the serine requirement was satisfied by 10^{-2} M-glycine in the absence of CO_2 (Table 1). The parent strain does not grow significantly in the absence of serine unless the glycine concentration is increased above the basal concentration of 5×10^{-4} M and the atmosphere enriched with CO_2. Such differences might be expected if the organism had been 'trained' to synthesize serine more efficiently. This would seem to have been the case since suspensions of the variant strain yielded twice the concentration of serine produced by the parent strain under the same conditions.

Table 1. *Response of parent and variant strains of* Leuconostoc mesenteroides *P* 60 *to glycine, serine and* CO_2

Basal medium *L* was supplemented with 10^{-6} M-*p*-AB, 5×10^{-4} M-glycine and 2×10^{-7} M-pyridoxal.

Additions to basal medium	Atmosphere	Growth at 44 hr. (EEL reading)	
		P 60	Variant
None	N_2	1·5	8·0
10^{-2} M-glycine		4·0	21·0
2×10^{-3} M-DL-serine		26·0	22·5
None	5 % (v/v) CO_2 + 95 % (v/v) N_2	1·0	16·0
10^{-2} M-glycine		26·5	22·5
2×10^{-3} M-DL-serine		29·5	23·5

As with the parent strain, formate did not replace CO_2 in stimulating growth of the variant. The growth-promoting effects of *p*-aminobenzoic acid, leucovorin (which replaced both *p*-aminobenzoic acid and CO_2) and pyridoxal in the presence and absence of serine were qualitatively identical with those observed with the parent strain.

The synthesis of serine by suspensions of Leuconostoc mesenteroides *P*60

Suspensions of the variant strain in solution *D* synthesized about 0·25 μmoles L-serine/ml. medium in 24 hr. when incubated at 37° either in air or in N_2 (Fig. 2). Glycine, glucose, formate, *p*-aminobenzoic acid and pyridoxal were all essential for synthesis (Table 2). Enrichment of the atmosphere with CO_2 had no effect upon serine formation (Tables 2 and 3). The requirement for glycine in concentrations of about 100 times those of the serine formed reflects the replacement of serine for growth of *Leuconostoc mesenteroides* P60.

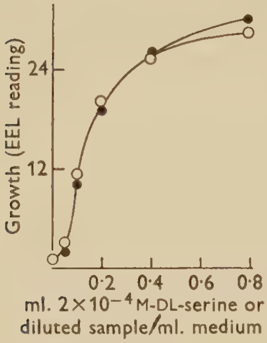

Fig. 1

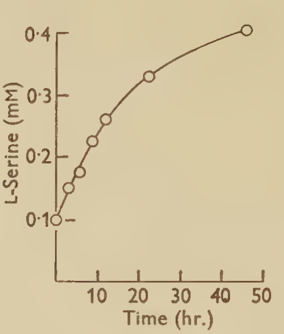

Fig. 2

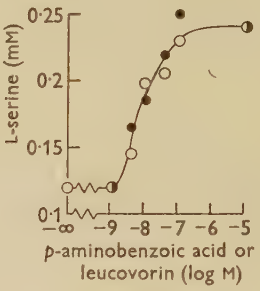

Fig. 3

Fig. 1. Response of *Leuconostoc mesenteroides* P60 to DL-serine (●—●) and to a typical acid-treated sample (○—○) containing serine synthesized by suspensions of *L. mesenteroides* P60.

Fig. 2. Rate of synthesis of L-serine by suspensions of the variant strain of *Leuconostoc mesenteroides* P60 in solution *D* incubated in N_2.

Fig. 3. Effect of *p*-aminobenzoic acid (●—●) or leucovorin (○—○) concentration on the synthesis of L-serine by suspensions of the variant strain of *Leuconostoc mesenteroides* P60 in solution *D*. Incubation was for 24 hr. at 37°.

Leucovorin, a mixture of the (+) and (−) L-isomers of calcium N^5-formyltetrahydropteroylglutamate pentahydrate (synthetic '*Leuconostoc citrovorum* factor') has about one half of the activity of *p*-aminobenzoic acid for the growth of *L. mesenteroides* P60 in the absence of serine (Lascelles *et al.* 1954). Leucovorin had the same activity as *p*-aminobenzoic acid for the synthesis of serine by suspensions of

Table 2. *Synthesis of serine by suspensions of the variant strain of* Leuconostoc mesenteroides *P*60

Incubation was in the basal solution *D* (with omissions as below) for 24 hr.

Omissions from basal solution	Atmosphere	L-Serine found (mM)
None	20 % (v/v) CO_2 + 80 % (v/v) N_2	0·25
None (heated control)		0·05
Glycine		0·0
Pyridoxal		0·03
Formate		0·03
p-AB		0·08
Glucose		0·0
None	N_2	0·25

L. mesenteroides P 60, variant strain (Fig. 3). Both compounds were optimal at 2×10^{-7} M and half optimal at 10^{-8} M. Though leucovorin contains only 50 % of the natural isomer (Cosulich, Smith & Broquist, 1952) it cannot be concluded that this isomer is twice as active as *p*-aminobenzoic acid for synthesis by the suspensions, as a difference of two in the activities of *p*-aminobenzoic acid and leucovorin would be within the limits of experimental error.

Table 3. *Synthesis of serine by suspensions of the variant strain of* Leuconostoc mesenteroides *P* 60

Organisms incubated in basal solution *D* minus *p*-AB for 24 hr. after pre-incubation where indicated.

Addition to basal solution	Atmosphere	L-Serine (mM) found when organisms Pre-incubated	Normal
10^{-6} M-*p*-AB	N_2	0·05	0·3
	20 % (v/v) CO_2 + 80 % (v/v) N_2	0·2	0·25
10^{-6} M-Leucovorin	N_2	0·2	0·25
	20 % (v/v) CO_2 + 80 % (v/v) N_2	0·2	0·23

Pteroylglutamic acid, which does not replace *p*-aminobenzoic acid in supporting growth of *Leuconostoc mesenteroides* P 60, was also unable to replace it for the synthesis of serine by suspensions of organisms. A concentration of 5×10^{-3} M-formate in the medium was necessary for maximum synthesis of serine though formate had no effect on the growth of the organism either in the presence or absence of serine. These observations parallel those with *Streptococcus faecalis* R (Lascelles & Woods, 1954). The synthetic ability of *L. mesenteroides* P 60 variant strain did not vary significantly with physiological age over the range 8–16 hr. growth in medium *C*. There was no destruction of added DL-serine when incubated with suspensions of the variant strain in solution *D*.

The chromatographic identification of serine

To confirm the results of microbiological assay the serine formed by suspensions of the variant strain of *Leuconostoc mesenteroides* P 60 was identified chromatographically on columns of Dowex-50 resin by a procedure like that described by Lascelles & Woods (1954) for a similar analysis of samples from *Streptococcus faecalis* R suspensions. Samples from systems incubated without *p*-aminobenzoic acid and without pyridoxal contained less than 15 % of the serine in the complete system, confirming results of microbiological assay.

The activity of suspensions of Leuconostoc mesenteroides *P* 60 *previously incubated in glucose*

The absence of any stimulation by CO_2 of serine production by suspensions, in contrast to its ability to replace serine for growth of *Leuconostoc mesenteroides* P 60, suggested that the harvested organisms might contain sufficient of some cofactor normally synthesized from CO_2. In case such a cofactor might be degraded by the organism and thus removed, organisms, grown as already described, were incubated in glucose and phosphate buffer (pH 6·9) before incubation in solution *D*. Suspensions of organisms treated in this way formed less serine, but the activity was largely restored by enriching the atmosphere with CO_2 or by replacing *p*-aminobenzoic acid by leucovorin (Table 3). Stimulation by CO_2 was most marked after 3 hr. pre-incubation.

The inhibition of serine synthesis by sulphathiazole

The synthesis of serine by suspensions of *Leuconostoc mesenteroides* P60 variant strain was competitively inhibited by sulphathiazole (Table 4). When *p*-aminobenzoic acid was replaced by leucovorin in solution *D* serine synthesis was no longer so inhibited by sulphathiazole at the concentrations tested (Table 5). Leucovorin non-competitively antagonized the inhibition by sulphathiazole of the growth of *Leuconostoc mesenteroides* P60 (Lascelles *et al.* 1954). These results suggest that a leucovorin-like compound is a product of the utilization of *p*-aminobenzoic acid by this organism.

Table 4. *Inhibition by sulphathiazole of the synthesis of serine by suspensions of the variant strain of* Leuconostoc mesenteroides *P* 60

Organisms incubated in a basal solution *D* (minus *p*-AB) for 24 hr.

p-AB added (M)	Sulphathiazole/*p*-AB ratio found for 50 % inhibition of synthesis
4×10^{-7}	1·8
10^{-6}	1·3
4×10^{-6}	1·6
4×10^{-5}	1·4

Table 5. *Reversal by leucovorin of sulphathiazole inhibition of serine synthesis by suspensions of the variant strain of* Leuconostoc mesenteroides *P* 60

Organisms incubated in basal solution *D* (minus *p*-AB) for 24 hr.

Sulphathiazole added (M)	Leucovorin required for 50 % of optimum synthesis (M)
—	$1·0 \times 10^{-8}$
10^{-6}	$2·5 \times 10^{-8}$
10^{-5}	$1·5 \times 10^{-8}$
10^{-4}	$3·0 \times 10^{-8}$

DISCUSSION

Though CO_2 and a relatively high concentration of glycine are essential to the growth of *Leuconostoc mesenteroides* P60 in the absence of serine, $^{14}CO_2$ is not incorporated into the serine of the organism. The simplest explanation, particularly in the light of evidence to be discussed in the following paper (Cross, 1960), is that CO_2 does not act as a donor of the β-carbon of serine. It is possible that exogenous CO_2 acts as a one-carbon donor in the initial stages of growth only until sufficient of some endogenous source of the β-carbon of serine is available. This seems unlikely, since formate does not satisfy the requirement of the organism for CO_2, even though it is a substrate for the conversion of glycine to serine by suspensions of *Leuconostoc mesenteroides* P 60 and is incorporated into serine by the growing organism. Further, optimum synthesis of serine from glycine by specially treated suspensions of the variant strain is only achieved in the presence of both CO_2 and formate.

Serine synthesis was studied in suspensions of the variant strain since it proceeded more actively with this variant than with the parent strain of *Leuconostoc mesenteroides* P 60. Serine is formed from glycine and formate provided that pyridoxal and either *p*-aminobenzoic acid or leucovorin are supplied. The system is analogous to that described by Lascelles & Woods (1954) with suspensions of *Streptococcus faecalis* R, the requirement for a folic acid group factor in their system being met by pteroylglutamic acid or leucovorin, either of which will also support growth of the organism. Maximum synthesis of serine by suspensions of *Leuconostoc mesenteroides* P 60 variant strain, which had been previously incubated in phosphate-buffered glucose solution, required CO_2 in addition. Presumably some factor essential for serine synthesis, be it substrate or enzyme, had been destroyed during this preliminary incubation. Leucovorin, for example, is destroyed under these conditions (Cross, 1960). The ability to form serine was restored when CO_2 was available. Perhaps conditions in these suspensions of pre-incubated organisms more closely paralleled those in cultures of the growing organism. The role of CO_2 in promoting serine synthesis in suspensions of the organism is examined further in the following paper (Cross, 1960).

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The Synthesis of Citrovorum Factor by *Leuconostoc mesenteroides* P 60

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SUMMARY

When incubated with *p*-aminobenzoic acid, formate and glucose, suspensions of a variant strain of *Leuconostoc mesenteroides* strain P 60 formed two factors possessing folic acid activity. One of these factors behaved like rhizopterin in supporting the growth of *Streptococcus faecalis* strain R but not of *Lactobacillus casei*. The other, like leucovorin, supported the growth of *Leuconostoc citrovorum*. The synthesis of 'citrovorum factor' was enhanced 5- to 20-fold by the presence of CO_2 in the gas phase. Sulphathiazole inhibited the synthesis of both factors, the inhibition being overcome competitively by *p*-aminobenzoic acid. Leucovorin was destroyed by suspensions of *L. mesenteroides* P 60 in phosphate-buffered glucose. The diminution of activity for *L. citrovorum* (decrease of citrovorum factor) was decreased when the incubation was carried out in a CO_2-enriched atmosphere. Ascorbic acid, which behaved similarly to CO_2 in partially preventing inactivation of leucovorin by suspensions of *L. mesenteroides* P 60 variant strain, also increased the synthesis of citrovorum factor from formate and *p*-aminobenzoic acid and partially satisfied the requirement of the growing *L. mesenteroides* P 60 for CO_2.

INTRODUCTION

Carbon dioxide will replace serine for the growth of *Leuconostoc mesenteroides* P 60 provided that glycine is present at relatively high concentration; yet, under these conditions, $^{14}CO_2$ is not incorporated into the serine synthesized (Cross, 1960). The growth of *L. mesenteroides* P 60 also occurs in the absence of both serine and CO_2, when the *p*-aminobenzoic acid in the medium is replaced by leucovorin (Lascelles, Cross & Woods, 1954). This suggested that the group of reactions in which the intervention of CO_2 is necessary might be those leading from *p*-aminobenzoic (*p*-AB) acid to the final coenzyme form of folic acid. The present work provides evidence of the dependence of the '*L. citrovorum* factor' (citrovorum factor) concentration in suspensions of a variant strain of *L. mesenteroides* P 60 upon the presence of carbon dioxide in the gas phase.

METHODS

Leuconostoc mesenteroides *P* 60. The two strains used were those used by Cross (1960). The variant strain was derived from *L. mesenteroides* P 60 (*Streptococcus equinis* P 60; ATCC 7881) by repeated subculture in the absence of added serine. The variant strain synthesizes serine more efficiently in growing cultures and in suspensions than does the parent strain. The media, conditions of growth and the method of assessment of growth were as described previously.

Suspensions of organisms deficient in *p*-aminobenzoic acid and in vitamin B_6 were also obtained and incubated as previously described. The complete solution for the synthesis of citrovorum factor normally contained: glucose, 10^{-2}M; sodium formate, 10^{-2}M; *p*-aminobenzoic acid, 10^{-5}M; phosphate buffer, pH 6·9, 10^{-1}M. In some experiments, in which the synthesis of serine and citrovorum factor were being observed concurrently, the reaction mixture (solution *D*) also contained: glycine, 2×10^{-2}M and pyridoxal, 10^{-6}M (Cross, 1960). Modifications in particular experiments are noted in the text.

In experiments in which the organisms were incubated in 2×10^{-2}M-glucose and 10^{-1}M-phosphate buffer (pH 6·9) before testing their synthetic ability, the procedure was again exactly as previously described (Cross, 1960).

Microbiological assay of citrovorum factor. The preparation of material for the assay of citrovorum factor and the assay with *Leuconostoc citrovorum* (ATCC 8081) was carried out as described by Lascelles & Woods (1952, 1954). Leucovorin was used as a standard and gave a dose–response curve similar to that of the sample under test (Fig. 1). The effective range of the assay was from 0·2 to 1·5 $\mu\mu$mole leucovorin with an accuracy of about ±10%. Results are expressed in terms of mμM-leucovorin.

Microbiological assay of 'Lactobacillus casei *factor*'. The preparation of material was carried out as for the citrovorum factor assay. The assay with *Lactobacillus casei* (ATCC 7469) was performed by the method of Nimmo-Smith, Lascelles & Woods (1948), slightly modified in that a final volume of 2·0 ml./tube was found adequate to obtain reproducible results. As in other growth experiments, extent of growth was estimated with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) using 0·25 in. tubes and a neutral density filter.

Microbiological assay of factors active for Streptococcus faecalis *R*. The material for assay was prepared as above. The test organism was *S. faecalis* Rogers (ATCC 8043) maintained as described by Lascelles & Woods (1954); the assay procedure was that of Lascelles & Woods (unpublished). Medium *G* of Lascelles & Woods (1954), modified by the exclusion of thymine and pteroylglutamic acid, and the addition of pyridoxine (1 mg./l.) was the basal medium. The latter, together with the test solution or pteroylglutamic acid as standard, was distributed to 1·9 ml. final volume in hard glass tubes (125 × 16 mm.), and autoclaved for 7 min. at 10 lb./sq.in. The inoculum was derived from a fresh stab culture in medium B_1 which was subcultured into medium B_2 (Nimmo-Smith *et al.* 1948) and incubated 18 hr. at 37°. This culture was diluted fifty-fold, and each tube inoculated with 0·1 ml. (about 10^7 organisms). Incubation was for 20–24 hr. at 37° in air.

Standards containing pteroylglutamic acid (1 to 16×10^{-10}M) were set up in each assay; the content of factors active for *Streptococcus faecalis* R is recorded as mμM equivalent pteroylglutamic acid by direct comparison of the response obtained at two concentrations with the dose–response curve given by synthetic pteroylglutamic acid.

The response curve for samples approximated to that for pteroylglutamic acid (PtG) at lower concentration (Fig. 2). The deviation at higher levels will be discussed in the text.

Chemicals. The pteroylglutamic acid was 'Folvite' (Lederle Laboratories, Pearl River, New York, U.S.A.). The source of all other materials, and the preparation and storage of stock solutions, were described by Lascelles *et al.* (1954).

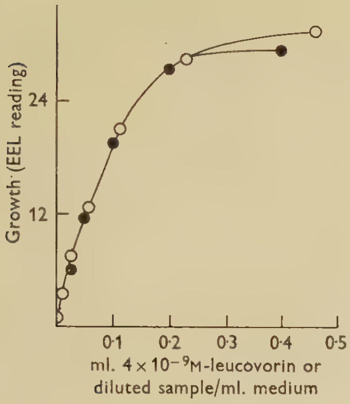

Fig. 1

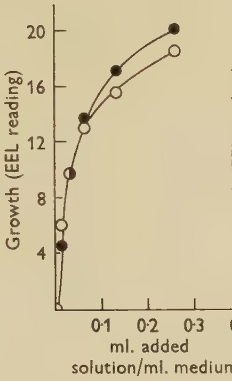

Fig. 2*a*

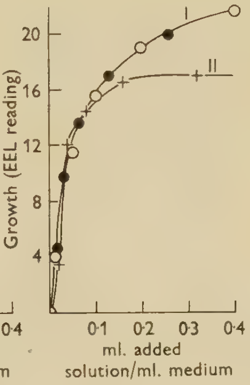

Fig. 2*b*

Fig. 1. Response of *Leuconostoc citrovorum* to leucovorin (●—●) and to a typical sample (○—○) containing active factor synthesized by suspensions of *L. mesenteroides* P 60 variant strain.

Fig. 2*a*. Response of *Streptococcus faecalis* R to pteroylglutamic acid (○—○) and to a typical sample (●—●) containing active factor synthesized by suspensions of *Leuconostoc mesenteroides* P 60 variant strain.

Fig. 2*b*. Response of *Streptococcus faecalis* R to I, N^{10}-formylpteroic acid (○—○) and a typical sample (●—●) containing active factor synthesized by suspensions of *Leuconostoc mesenteroides* P 60 variant strain; and II, pteroic acid (+—+). N^{10}-formylpteroic and pteroic acids were added, as 4×10^{-9} M and 4×10^{-8} M solutions respectively diluted from 10^{-6} M solutions in 'boiled control' samples. The sample was added as a 1 in 60 dilution of the incubated system.

RESULTS

The synthesis of citrovorum factor by suspensions of Leuconostoc mesenteroides *P 60 variant strain*

Lascelles & Woods (1954) showed that during the synthesis of serine from glycine and formate by suspensions of *Streptococcus faecalis* R, there was a concurrent formation of a factor or factors active for the growth of *L. citrovorum.* A similar appearance of this factor attended the production of serine by suspensions of *L. mesenteroides* P 60; glucose, formate and *p*-aminobenzoic acid were essential substrates (Table 1; Figs. 3–5).

The growth of *Leuconostoc mesenteroides* P 60 in the absence of serine and leucovorin is dependent on the enrichment of the atmosphere with CO_2 (Lascelles *et al.* 1954). No effect of such a gas phase on the synthesis of serine by suspensions of *L. mesenteroides* P 60 variant stain were detected, but the increase in citrovorum factor synthesis was considerable, varying from 5- to 20-fold in a number of experiments.

A maximum effect of CO_2 was observed at a partial pressure of 20 % atmospheric (Fig. 6). The enhancement of citrovorum factor synthesis cannot be ascribed to a

Table 1. *Synthesis of factors with folic acid activity by suspensions of* Leuconostoc mesenteroides *P*60 *variant strain*

Incubation was in the basal solution *D* (with omissions as below) for 24 hr.

Omissions from basal solution	Gas phase	'*S. faecalis* R factor' found (mμM)	'Citrovorum factor' found (mμM)
None	20 % (v/v) CO_2 + 80 % (v/v) N_2	350	40
None (heated control)		5	1
Glycine		300	40
Pyridoxal		350	40
Formate		20	3
p-AB		5	1
Glucose		30	3
None	N_2	350	4

change in the pH value of the medium (Table 2). Over a range of initial pH values from 7·1 to 6·1 the decrease in pH during incubation in N_2 was about 0·3–0·6 of a unit. This increased to between 0·5 and 0·8 of a pH unit when incubation was in 20 % (v/v) CO_2 + 80 % (v/v) N_2. Though citrovorum factor formation was apparently increased by an acid environment, the synthesis in N_2 at initial pH 6·1 was only about 40 % of that in 20 % (v/v) CO_2 + 80 % (v/v) N_2 at initial pH 7·1. The increased synthesis in more acid media cannot account for the carbon dioxide effect.

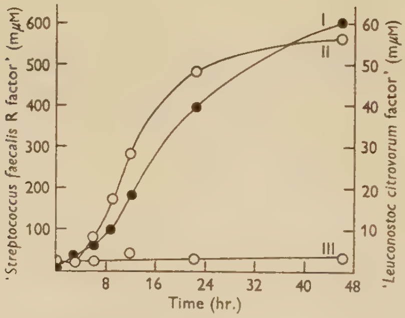

Fig. 3

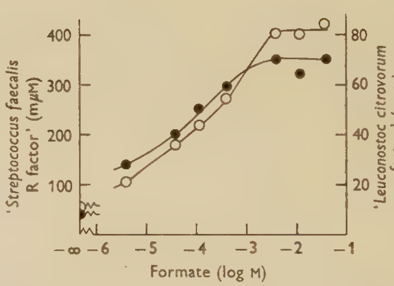

Fig. 4

Fig. 3. Rates of synthesis by suspensions of the variant strain of 'citrovorum factor' (○—○) and '*Streptococcus faecalis* R factor' (●—●) in solution *D*. Curves I and III were obtained by incubation in N_2; curve II by incubation in 20 % CO_2 in N_2

Fig. 4. Effect of formate concentration on the synthesis of 'citrovorum factor' (○—○) in 20 % CO_2 in N_2, and '*Streptococcus faecalis* R factor' (●—●) in N_2 by suspensions of the variant strain. Incubation for 24 hr. in solution *D* omitting pyridoxal and glycine.

It seemed possible that CO_2 might be involved in the formation of the glutamic acid moiety of citrovorum factor. Both DL- and L-glutamic acid were inactive however, over the concentration range 10^{-1} to 10^{-4}M.

The glucose concentration is also a critical factor in the production of citrovorum factor; peak synthesis occurred at *c.* 10^{-2}M-glucose.

Table 2. *Effects of* pH *value and* CO_2 *on synthesis of folic acid group factors by suspensions of* Leuconostoc mesenteroides *P*60 *variant strain*

Suspensions incubated in solution *D* minus glycine and pyridoxal for 24 hr.

pH value of suspension			'Citrovorum factor' (mμM) found. After incubation in		'*S. faecalis* R factor' (mμM) found. After incubation in
	After incubation in				
Initial	N_2	CO_2	N_2	CO_2	N_2
7·1	6·6	6·3	4·5	33·0	380
6·7	6·4	6·2	7·0	33·0	125
6·3	5·9	5·6	10·5	35·5	75
6·1	5·5	5·3	13·5	31·5	55

As for maximum serine synthesis, 5×10^{-3} M-formate was the minimum concentration which gave an optimum yield of citrovorum factor (Fig. 4). However, production of the latter increased with *p*-aminobenzoic acid concentration up to 10^{-5} M, a hundred times that required for most rapid formation of serine (Fig. 5). Pteroylglutamic acid was not converted to citrovorum factor by suspensions.

The ability of the organisms to synthesize citrovorum factor varied with their physiological age. For best activity the organisms for suspension were harvested after 10–11 hr. as previously described (Cross, 1960).

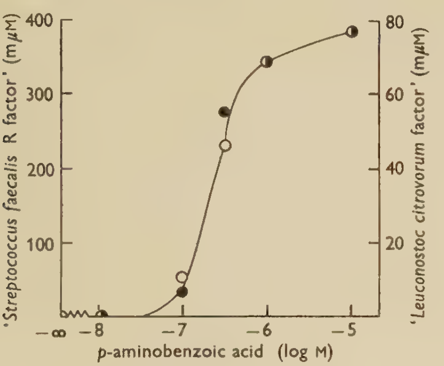

Fig. 5

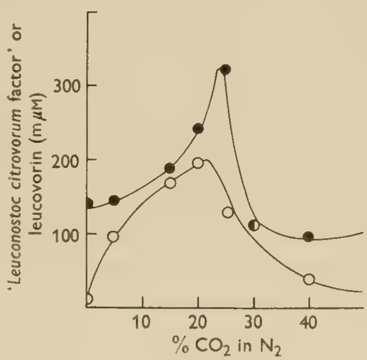

Fig. 6

Fig. 5. Effect of *p*-aminobenzoic acid concentration on the synthesis of 'citrovorum factor' (●—●) (incubation in 20% CO_2 in N_2) and 'Streptococcus faecalis R factor' (○—○) (incubation in N_2) by suspensions of the variant strain incubated in solution *D* omitting glycine and pyridoxal, for 24 hr.

Fig. 6. Effect of partial pressure of CO_2 on the destruction of leucovorin and the synthesis of 'citrovorum factor' by the variant strain. Leucovorin remaining (●—●) from an initial concentration of 1000 mμM after incubation with organisms suspended in phosphate buffer and 10^{-2} M-glucose; 'citrovorum factor' synthesized (○—○) in solution *D* omitting glycine and pyridoxal. Incubation was for 24 hr. at 37° in both cases.

The synthesis of factors active for Streptococcus faecalis *R by suspensions of* Leuconostoc mesenteroides *P*60, *variant strain*

The supernatant fluids from suspensions of the variant strain in solution *D* incubated in N_2 (which contained serine and a little citrovorum factor) were also active in supporting the growth of *S. faecalis* R, and in replacing pteroylglutamic acid

The concentration of active factor in terms of equivalent pteroylglutamic acid was about one hundred times that of the citrovorum factor present, in terms of equivalent leucovorin (Fig. 3). Since it is likely that the (−)-L-isomer of leucovorin (the active isomer) has activity for *S. faecalis* R equal to that of pteroylglutamic acid (Broquist *et al.* 1952; Cosulich, Smith & Broquist, 1952), and the citrovorum factor synthesized by *L. mesenteroides* P 60 would appear to be at least closely related to leucovorin, it seems that this activity for *S. faecalis* R cannot be ascribed to the small quantity of citrovorum factor present. Furthermore, suspensions incubated in 20 % CO_2 yielded the same, or in some experiments a lower, concentration of '*S. faecalis* R factor' than those incubated in N_2, in contrast to the greater activity of the former supernatant fluids for *L. citrovorum* (Table 1).

In other respects the requirements for '*Streptococcus faecalis* R factor' synthesis are identical to those for formation of citrovorum factor (Table 1; Figs. 3–5). Glucose, *p*-aminobenzoic acid and formate were essential components of the system; omission of glycine and pyridoxal from solution *D* had no significant effect. The production of '*S. faecalis* R factor', like that of citrovorum factor, reaches a maximum at 10^{-5}M-*p*-aminobenzoic acid and 5×10^{-3}M-formate. The synthesis, however, increases with glucose concentration up to 10^{-1}M; there is no peak activity at a lower concentration as with citrovorum factor.

Neither after incubation in CO_2 nor in N_2 was any activity for *Lactobacillus casei* demonstrable beyond that which could be ascribed to citrovorum factor in the samples. The response of *Streptococcus faecalis* R to the preparations was not due, therefore, to the presence of pteroylglutamic acid, which also supports growth of *L. casei* (Broquist *et al.* 1952).

The response of *Streptococcus faecalis* R to the supernatant fluid from a suspension of *Leuconostoc mesenteroides* P 60 variant strain in glucose, phosphate buffer, formate and *p*-aminobenzoic acid incubated in N_2 was compared to the response to pteroic acid and rhizopterin (Fig. 2*b*). The pteroic acid and rhizopterin added as standards were diluted from 10^{-6}M solutions in supernatant fluids from 'boiled control' suspensions of the variant strain in the above medium. The dose/response curve for the active samples much more nearly approached that for rhizopterin than that for pteroic acid.

The activity of suspensions of Leuconostoc mesenteroides *P* 60 *previously incubated in glucose*

After pre-incubation, *Leuconostoc mesenteroides* P 60 variant strain incubated in solution *D* did not yield significant quantities of citrovorum factor whether CO_2 was present or not; however, their ability to synthesize '*Streptococcus faecalis* R factor' was not impaired (Table 3).

The response of Leuconostoc mesenteroides *P* 60 *to factors formed by suspensions of the variant strain*

It was not possible to draw any conclusions from direct tests of the ability of samples to support the growth of *Leuconostoc mesenteroides* P 60 since these samples contained relatively high concentrations of *p*-aminobenzoic acid which is the most potent member of the folic acid group known for this organism. However, the relative efficiencies of samples in antagonizing sulphathiazole-inhibition were as

Table 3. *Synthesis of folic acid group factors by suspensions of* Leuconostoc mesenteroides *P 60 variant strain*

Organisms incubated in basal solution *D* for 24 hr. after pre-incubation where indicated.

Gas phase	'Citrovorum factor' (mμM) found when organisms		'*S. faecalis* R factor' (mμM) found when organisms	
	Pre-incubated	Normal	Pre-incubated	Normal
N_2	0·2	0·2	550	550
20% (v/v) CO_2 + 80% (v/v) N_2	0·2	8·2	550	550

might be expected if a rhizopterin-like compound were formed by incubating suspensions in N_2, together with a leucovorin-like compound when incubation was in an atmosphere enriched with CO_2 (Table 4). Sulphathiazole inhibited growth of the parent strain in the presence of a sample showing activity for *Streptococcus faecalis* R equivalent to $2{\cdot}2 \times 10^{-8}$ M-pteroylglutamic acid; but did not inhibit, even at 10^{-3} M-sulphathiazole, a sample (from incubation in 20% CO_2) which showed also citrovorum factor equivalent to $4{\cdot}2 \times 10^{-9}$ M-leucovorin. Rhizopterin is inactive for the growth of *L. mesenteroides* P 60 (Lascelles *et al.* 1954).

Table 4. *Antagonism of sulphathiazole-inhibition of the growth of* Leuconostoc mesenteroides *P 60 by factors of the folic acid group*

Leuconostoc mesenteroides P 60 was grown in medium *L* supplemented with 5×10^{-4} M-glycine, 10^{-3} M-DL-serine and 2×10^{-7} M-pyridoxal in air. The added samples were derived from incubated suspensions of the variant strain prepared for assay in the usual way.

Sample A (from incubation in N_2) contains '*Streptococcus faecalis* R factor' equivalent to $2{\cdot}2 \times 10^{-8}$ M-PtG and 'citrovorum factor' equivalent to $0{\cdot}1 \times 10^{-9}$ M-leucovorin.

Sample B (from incubation in CO_2) contains '*S. faecalis* R factor' equivalent to $1{\cdot}8 \times 10^{-8}$ M-PtG and 'citrovorum factor' equivalent to $4{\cdot}2 \times 10^{-9}$ M-leucovorin.

Sample added (% (v/v) of final volume)	Sulphathiazole added (M)	Sample added A — Growth (EEL reading at 44 hr.)	Sample added B — Growth (EEL reading at 44 hr.)
0·5	—	24·0	21·5
0·5	10^{-3}	8·0	18·0
1·0	—	21·5	23·5
1·0	10^{-3}	8·5	24·0
2·0	—	20·5	24·0
2·0	10^{-3}	10·5	30·5

The inhibition of the synthetic ability of suspensions of the variant strain by sulphathiazole

Like the synthesis of serine (Cross, 1960), the synthesis of citrovorum factor and '*Streptococcus faecalis* R factor' by suspensions of *Leuconostoc mesenteroides* P 60 variant strain was inhibited by sulphathiazole; each inhibition was competitively antagonized by *p*-aminobenzoic acid (Table 5). '*S. faecalis* R factor' formed by suspensions of *L. mesenteroides* P 60 variant strain did not annul sulphathiazole-inhibition of the growth of *L. mesenteroides* P 60 in a non-competitive manner. Yet the Streptococcus factor is the product of a sulphathiazole-inhibited reaction in *L. mesenteroides* P60. The simplest interpretation of these facts is that the Streptococcus factor cannot support the growth of *L. mesenteroides* P60. This is again in accordance with indications that the Streptococcus factor is a rhizopterin-like compound.

Table 5. *Inhibition by sulphathiazole of the synthesis of folic acid group factors by suspensions of* Leuconostoc mesenteroides *P*60 variant strain

Organisms incubated in solution *D* minus *p*-AB for 24 hr.

p-AB added (M)	Sulphathiazole/*p*-AB ratio required for 50 % inhibition of synthesis of	
	'Citrovorum factor'	'*S. faecalis* R factor'
4×10^{-7}	2·0	2·0
10^{-6}	1·6	1·5
4×10^{-6}	1·6	1·3
4×10^{-5}	1·0	1·3

The inactivation of leucovorin by suspensions of Leuconostoc mesenteroides *P*60 *variant strain*

The decrease of activity of leucovorin (citrovorum factor) added to suspensions of *Leuconostoc mesenteroides* P60 variant strain was rapid. Carbon dioxide partially prevented the inactivation of leucovorin during incubation, maximum inhibition occurring in atmospheres containing 20–25 % CO_2. This is a partial pressure which approximates to that required for maximum *de novo* formation of citrovorum factor (Fig. 6). Of the leucovorin originally present only 7 % remained after incubation with *L. mesenteroides* P60 variant strain in N_2 whilst 16 % remained in 25 % (v/v) CO_2+75 % (v/v) N_2. Leucovorin activity was not lost by incubation at 10^{-6}M with suspensions inactivated by heating at the outset of the experiment, nor in the course of preparation of samples for assay.

The inactivation of leucovorin was considerable even in the absence of glucose, but was greatly accelerated in its presence. A critical increase in this enhancement of the degradation occurred at about 10^{-2}M-glucose; this might explain the diminution in apparent citrovorum factor synthesis with increasing glucose concentration which occurred at this concentration of glucose, resulting in the peak already described.

The replacement of CO_2 *by ascorbic acid*

Carbon dioxide has the dual capacity of stimulating serine synthesis (Lascelles *et al.* 1954; Cross, 1960) and of preventing leucovorin inactivation by *Leuconostoc mesenteroides* P60. Any hypothesis about a relationship between these two activities would be supported if another compound which produced one of the phenomena also produced the other. With such a possibility in view tests were carried out with L-ascorbic acid which has been reported to increase citrovorum factor production by suspensions and extracts of *Streptococcus faecalis* R (Broquist *et al.* 1953; Nichol, 1954). When examined L-ascorbic acid (10^{-2}M) was found to stimulate synthesis of citrovorum factor from formate and *p*-aminobenzoic acid and partially prevented inactivation of 10^{-6} M-leucovorin by suspensions of *L. mesenteroides* P 60 variant strain, though the effect was not so marked as that produced by CO_2 (Table 6). It was also found that (*a*) recovery of 5×10^{-8} M-leucovorin from 'boiled controls' was almost complete in the presence or absence of ascorbic acid, and that (*b*) L-ascorbic acid had little effect when it was present only during the preparation of the samples for assay after incubation.

Table 6. *Effect of ascorbic acid and* CO_2 *on the synthesis and degradation of 'citrovorum factor' by suspensions of* Leuconostoc mesenteroides *P 60 variant strain*

Incubation in 10^{-2} M-glucose and 10^{-2} M-phosphate buffer for 24 hr. In the flasks in which 'citrovorum factor' synthesis was measured 10^{-6} M-*p*-AB and 10^{-2} M-formate were also present; in the flasks in which leucovorin degradation was measured, 10^{-6} M-leucovorin was added before incubation.

		'Citrovorum factor' (mμM) found	
L-Ascorbic acid (M) added	Gas phase	Synthesized from formate and *p*-AB	Remaining from 10^{-6} M initial leucovorin
—	N_2	1·4	9·5
10^{-2}	N_2	9·3	45·2
—	20 % CO_2 in N_2	40·5	80·0
10^{-2}	20 % CO_2 in N_2	68·0	86·0

Ascorbic acid partially replaced CO_2 for the growth of *Leuconostoc mesenteroides* P 60 in the absence of serine, but growth only reached one-third of that attained in 5 % (v/v) CO_2 + 95 % (v/v) N_2. The activity of L-ascorbic acid was confirmed by its stimulatory action when the organism was grown in the absence of serine with suboptimal concentrations of leucovorin or CO_2 (Table 7). When medium *L* was supplemented with 10^{-3} M-DL-serine no such stimulation occurred.

Table 7. *Effect of ascorbic acid on the growth of* Leuconostoc mesenteroides *P 60*

Growth in medium *L* supplemented with 5×10^{-4} M-glycine, 2×10^{-7} M-pyridoxal and 10^{-6} M-*p*-AB; and with and without 5×10^{-3} M-L-ascorbic acid.

		Growth (EEL reading in 44 hr.)	
Additions to basal medium	Gas phase	Without ascorbate	With ascorbate
—	N_2	1·0	7·5
—	0·5 % CO_2 in N_2	6·5	18·5
—	5·0 % CO_2 in N_2	20·0	22·5
10^{-8} M-leucovorin	N_2	14·5	22·5
10^{-3} M-DL-serine	N_2	22·5	24·0

DISCUSSION

When incubated with *p*-aminobenzoic acid and formate suspensions of a variant strain of *Leuconostoc mesenteroides* P 60 form two factors having folic acid activity. *p*-Aminobenzoic acid is also required for the synthesis of serine (Cross, 1960), and these factors are formed concurrently with serine when glycine and pyridoxal are also provided. Though some form of folic acid is clearly required for serine synthesis by this organism, the present experiments provide no evidence of the identity of the final coenzyme form (CoF).

The two factors formed in the suspensions have been tentatively identified as rhizopterin and leucovorin on the basis of their activities for various micro-organisms. It is unlikely that either of these factors is even an intermediate in the synthesis of CoF from *p*-aminobenzoic acid. Rhizopterin is transformed to citrovorum factor by suspensions of *Streptococcus faecalis* R (Hendlin, Koditschek & Soars, 1953), but it does not support the growth of *Leuconostoc mesenteroides* P60. It is improbable that this inactivity is due to the impermeability of the cells to the factor, since leucovorin and tetrahydropteroyl-glutamic acid, both containing a pteridine residue, albeit reduced, support growth of the organism (Lascelles *et al.* 1954; Cross, 1956). Leucovorin, likewise, is inactive for a number of micro-organisms, and probably does not mediate in the main pathway to CoF (Woods, 1954). Nevertheless, it may be a sufficiently close by-product of CoF metabolism for its formation to give a measure of the synthesis of the coenzyme.

An inverse quantitative relationship exists between the requirements for leucovorin and glycine for the growth of *Leuconostoc mesenteroides* P60 in the absence of serine, just as it does between CO_2 and glycine (Lascelles *et al.* 1954). Leucovorin will also satisfy the requirement for CO_2 of suspensions of the pre-incubated cells synthesizing serine (Cross, 1960). One interpretation of these observations is that CO_2 maintains a concentration of CoF sufficient for the synthesis of serine. In support of this view it is now found that the formation of citrovorum factor in suspensions of the variant strain of *L. mesenteroides* P60 is enhanced by CO_2. If CO_2 is essential for the formation of CoF, then CO_2 should be necessary for the synthesis of serine. But optimum concentrations of serine are formed by suspensions of the freshly harvested variant strain in an atmosphere of N_2 (Cross, 1960). Again, unless *p*-aminobenzoic acid has some function in bacterial metabolism apart from the synthesis of CoF, *L. mesenteroides* P60 should fail to grow in atmospheres devoid of CO_2 unless the requirement for *p*-aminobenzoic acid were also eliminated. However, growth of the organism in the presence of serine occurs only when *p*-aminobenzoic acid is supplied, and yet it proceeds in N_2 (Lascelles *et al.* 1954).

Carbon dioxide partially inhibits the inactivation of leucovorin by suspensions of the variant strain of *Leuconostoc mesenteroides* P60. If this inactivation reflects a loss of CoF, then CO_2 may exert its influence on CoF concentration, and therefore on serine synthesis, by inhibiting degradation of the coenzyme. It is assumed that the CoF concentration is sufficiently high, even in the absence of CO_2, for the synthesis of serine by freshly harvested suspensions, and for the requirements of the organism growing in the presence of serine. On the other hand, it is insufficient, in the absence of CO_2, for the synthesis of serine either by growing organisms or by suspensions of organisms previously incubated in phosphate-buffered glucose. In fact no citrovorum factor formation has been detected in suspensions of the pre-incubated organisms, although serine is synthesized under these conditions. It may be that the assay is not sensitive enough to detect small quantities of citrovorum factor consonant with serine synthesis. The hypothesis that the parts played by CO_2, in serine synthesis, in citrovorum factor synthesis and in leucovorin degradation are closely related in *L. mesenteroides* P60, receives some support from the fact that ascorbic acid can partially replace CO_2 in each of these roles.

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A Study of the Factors Controlling Lactose-Fermentation in the Coliform and Paracolon Groups

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SUMMARY

Detailed study of a representative paracolon strain showed that the delayed fermentation of 1 % lactose coincided with the development of lactose-fermenting mutants, and that the essential difference between the normal non-lactose-fermenting (lac$^-$) organisms and the lactose-fermenting (lac$^+$) mutants was one of decreased permeability for lactose. Both types of organism showed the same degree of β-galactosidase activity when grown on lactose-free media; the permeability of the lac$^-$ organisms was increased by raising the concentration of lactose in the medium. On the assumption that permeability is governed by a specific permease which is genetically distinct from β-galactosidase, a hypothetical scheme based on these two factors together with the presence or absence of hydrogenlyase suggested the existence of six phenotypes among the paracolon bacilli. In an examination of 51 naturally occurring paracolon strains four of these phenotypes were demonstrated. Since the lactose-fermenting paracolon organisms differ from coliform bacilli merely in being less permeable to 1 % lactose, whereas the non-lactose-fermenting organisms differ from both the other groups in lacking β-galactosidase activity, it is suggested that the term paracolon should not be applied to this last group but should be restricted to the late-lactose-fermenters. Alternatively, these could be classed within the coliform group.

INTRODUCTION

The paracolon bacilli constitute a heterogeneous collection of organisms which are intermediate between the coliform bacilli and the salmonellas, shigellas, and other classified members of the Enterobacteriaceae. The heterogeneous nature of the paracolon group raises problems of considerable difficulty when attempts are made to classify and subdivide it. Various schemes based upon biochemical reactions and antigenic constitution have been presented but none has proved to be wholly satisfactory. The paracolon bacilli can be divided into two broad groups: the non-lactose-fermenting strains, and those strains which ferment 1 % lactose slowly. These groups can be further subdivided into aerogenic and anaerogenic types. Although the non-lactose-fermenting strains form a fairly compact group which is readily distinguishable from the coliforms and the classified non-lactose-fermenting organisms, opinions differ about the classification and nomenclature of the late-lactose-fermenting strains.

It is generally accepted that in the majority of instances delayed fermentation of lactose is dependent upon the development of sufficient lactose-fermenting (lac$^+$) mutants which occur spontaneously within the bacterial population (Lewis, 1934).

During recent years attention has been focused upon the fermentative differences between the normal non-lactose-fermenting (lac^-) organisms and the lac^+ mutants, and it would appear that the essential distinction is one of differences in permeability to lactose, and not of lactase (β-galactosidase) activity (Deere, 1939). Cohen & Monod (1957) described a specific enzyme-like system, which they named 'permease', which controls penetration of galactosides to the galactosidase site; these authors claimed that permease and β-galactosidase are genetically and functionally distinct. Further studies confirming these findings were reported by Cohn & Horibata (1959*a*, *b*, *c*). Lowe & Evans (1957), investigating a number of strains of late-lactose-fermenting paracolon bacilli, found that rapid fermentation of lactose occurred when the concentration of lactose was increased. The results obtained suggested that, although the normal lac^- organisms of the strains examined were impermeable to 1 % lactose, this barrier of impermeability could to some extent be overcome by increasing the concentration of lactose (see also Cohen & Monod, 1957). The present communication describes experiments undertaken to confirm and extend these findings and to attempt to clarify the classification of the paracolon bacilli based solely upon differences in the factors which control active fermentation of lactose.

METHODS

Organisms. The representative strain of test organism used in the preliminary experiments was originally obtained from the National Collection of Type Cultures (NCTC 8827), and will be referred to as culture E.C. 8. Fermentation of 1 % (w/v) lactose peptone water by this organism occurs after incubation for 3 days and coincides with the appearance of detectable lac^+ mutants. Colonies growing on 1 % lactose peptone agar produce typical lactose-fermenting papillae after incubation for 3 days. The spontaneous mutant count is approximately one lac^+ mutant organism to 100,000 normal lac^- organisms.

Strains of *Escherichia coli* and *Salmonella typhimurium* or *Proteus rettgeri* were put up in parallel with all experiments to act as controls of the media.

Media. The liquid media used were chemically defined and consisted of the following ingredients: $NH_4H_2PO_4$, 0·4 g.; Na_2HPO_4, 1·2 g.; lactose, 1 or 5 g.; glass-distilled water, 100 ml. All reagents were of analytical purity. Both media were sterilized by Seitz filtration. The solid media were prepared by mixing equal amounts of the appropriate double-strength liquid medium and sterile molten aqueous agar containing neutral red as the indicator. All media were at pH 7·2.

Procedure. Colonies of paracolon culture E.C. 8 grown on peptone agar were suspended in sterile distilled water and a suitable dilution made to give from one to five well separated colonies per plate on 1 % and 5 % lactose basal agar. Basal agar with the lactose omitted was included as a control against lactose-utilization. The inoculated plates were incubated at 37° for 10 days; each day the diameters of ten selected colonies were measured and these colonies were all inspected for colour and for the appearance of lactose-fermenting papillae (see Table 1).

A heavy inoculum was introduced into 100 ml. volumes of basal liquid containing, respectively, 1 %, 5 %, and no lactose. Samples (1 ml.) were withdrawn daily and tenfold dilutions made in distilled water. From each dilution 0·02 ml. was spread on 1 % lactose + 0·2 % peptone agar containing neutral red, and on nutrient agar. The duplicate series of plates were incubated at 37° and the resulting colonies counted after 24 hr. Similar total colony counts were obtained on both media. The presence of lac^+ mutant cells was readily detected on the lactose + peptone agar, and a proportion of these lactose-fermenting colonies were tested daily for ability to ferment 1 % lactose peptone water within 24 hr. Where the numbers of lac^+ mutant organisms did not exceed the expected mutant count (one lac^+ organism to 10^5 lac^- organisms) or where no lac^+ mutants were detected, a negative result was recorded (see Table 2).

Assay of β-ᴅ-galactosidase. The method was based upon colorimetric determination of *o*-nitrophenol liberated from the chromogenic substrate *o*-nitrophenyl-β-ᴅ-galactopyranoside (ONPG; L. Light and Co. Ltd., Colnbrook, Bucks) when in contact with whole organisms treated with benzene (Lederberg, 1950; Rotman, 1958). The lac^- normal and lac^+ mutant organisms of culture E.C. 8 were each grown on 1 % peptone agar and on 1 % peptone agar containing, respectively, 1 % lactose, 5 % lactose or 1 % glucose. The organisms were harvested after incubation for 24 hr. Each batch of organisms was washed twice in 0·01 ᴍ-sodium phosphate (Na_2HPO_4) buffer pH 7·5 and standardized by packed-cell volume to 0·25 ml.; 35 ml. phosphate buffer and 1·75 ml. benzene were added to each batch of organisms. The mixtures were shaken vigorously for 5 min. and then stored at 4° for 24 hr. Similar preparations of *Proteus rettgeri* and *Escherichia coli* were included as negative and positive controls of β-galactosidase.

The mixtures were transferred to the 37° water bath and, when temperature equilibrium had been reached, 35 ml. of 0·04 ᴍ-ONPG in sodium phosphate buffer were added to each batch of organisms. Samples (9 ml.) were withdrawn at 5 min. intervals and 1 ml. ᴍ-sodium carbonate was added to stop the reaction. The samples were centrifuged at 4000 rev./min. for 20 min. and the clear supernatant fluids used for the colorimetric determinations. The EEL portable colorimeter was used for all colorimetric determinations, with the OB 2 blue filter. Control blanks consisted of duplicate batches of benzene-treated organisms with the addition of equal volumes of sodium phosphate buffer. The ONPG control was prepared by adding 4 ml. sodium phosphate buffer and 1 ml. of ᴍ-sodium carbonate to 5 ml. of 0·04 ᴍ-ONPG. The colorimetric readings were converted into mg. *o*-nitrophenol liberated/100 ml., by comparing with standardized readings previously made against known concentrations of *o*-nitrophenol.

RESULTS

The results summarized in Tables 1 and 2 show the difference between the utilization of 1 % and 5 % lactose. In the agar series (Table 1) the increase in rate of colony growth on 1 % lactose agar coincided with the appearance of lactose-fermenting papillae. There was usually only one papilla associated with each colony, and the papilla seemed to be entirely responsible for the increase in colony size and for the development of an acid reaction throughout the colony. Colonies growing on 5 % lactose agar gave a progressive increase in size and acid reaction which was not dependent upon the development of papillae. Minute papillae were detected on the eighth day but these, when picked, failed to ferment 1 % and 5 % lactose peptone water within 24 hr. Colonies of *Salmonella typhimurium* developed on all these types of agar in a manner similar to that of culture E.C. 8 on the non-lactose basal agar.

Table 1. *Growth of culture E.C. 8 on basal agar containing* 1 % *and* 5 % *lactose showing colony size and development of lactose-fermenting papillae*

	Period of incubation (days)													
	1	2	3	4	6	8	10	1	2	3	4	6	8	10
Lactose (%)	Diameter of colony (mm.)							Appearance of lactose-fermenting papillae						
1	0·1	0·2	0·4	0·6	0·9	1·3	1·7	–	–	(+)	+	+	+	+
5	0·2	0·6	1·0	1·5	1·9	2·4	3·0	–	–	–	–	–	(+)	(+)
Nil	0·1	0·2	0·3	0·4	0·5	0·6	0·7	–	–	–	–	–	–	–

(+) = Papillae of minute size; + = well-developed papillae.

The lactose liquid media gave results (Table 2) similar to those obtained with the lactose agar series. Although there is evidence of slight utilization of 1 % lactose during the first 3 days, a considerable population increase and the development of acid coincided with the appearance of lac$^+$ mutants. In the 5 % lactose medium a progressive increase in population and acid-production occurred which was not dependent upon the development of detectable lac$^+$ mutants. The strain of *Salmonella typhimurium* developed in all three media in a similar manner to that of culture E.C. 8 in the lactose-free basal liquid.

Table 2. *Growth of culture* E.C. 8 *in basal liquid medium containing* 1 % *and* 5 % *lactose showing acid-production and the development of lactose-fermenting mutants*

	Basal medium								
Period of incubation (days)	Containing no lactose			Containing 1 % lactose			Containing 5 % lactose		
	N	F	pH	N	F	pH	N	F	pH
0	0·4	–	7·4	0·8	—	7·4	0·3	–	7·4
1	2·2	–	7·4	3·5	—	7·4	50	(–)	7·2
2	6·4	(–)	7·4	14·5	(–)	7·4	90	(–)	7·0
3	4·8	(–)	7·4	34	(–)	7·4	328	(–)	6·6
4	1·8	–	7·4	52	0·1	7·4	492	(–)	6·0
5	0·9	–	7·4	143	21	7·2	400	(–)	5·8
6	0·5	–	7·4	250	111	7·0	336	(–)	5·8
7	0·3	–	7·4	227	143	6·8	300	(–)	5·8
8	0·14	–	7·4	240	140	6·6	283	(–)	5·8
9	0·08	–	7·4	234	144	6·4	220	(–)	5·8
10	0·04	–	7·4	192	180	6·0	150	(–)	5·8

N = number of viable non-lactose-fermenting organisms (millions/ml.); F = number of viable lactose-fermenting mutants (millions/ml.); – = no lac$^+$ mutants detected; (–) = lac$^+$ mutants detected but not exceeding the expected mutant count (one lac$^+$ organism to 10^5 lac$^-$ organisms).

The β-galactosidase activity of the lac$^-$ normal and lac$^+$ mutants of culture E.C. 8 is shown in Fig. 1. The galactosidase activity of both types of organism when grown on peptone agar was similar, and depression of enzyme activity was apparent with the glucose-grown organisms. The normal lac$^-$ organisms when grown on 1 % lactose showed increased activity, and this was further increased when they were grown on 5 % lactose. The galactosidase activity obtained with the lac$^+$ mutants grown on 1 % or 5 % lactose was twice that of the lac$^-$ organisms grown on 5 %

lactose. The results suggest that the β-galactosidase activity of the lac^- organisms and lac^+ mutants when grown on peptone agar and glucose agar was similar and of a constitutive nature. It would also appear that the normal lac^- organisms are not wholly impermeable to 1 % lactose, and that substrate penetration is increased by higher concentrations of lactose. The essential fermentative difference, therefore, between the normal lac^- organisms and the lac^+ mutants of culture E.C. 8 is one of variable permeability to lactose and not of β-galactosidase content. These results are in agreement with the original findings of Deere (1939).

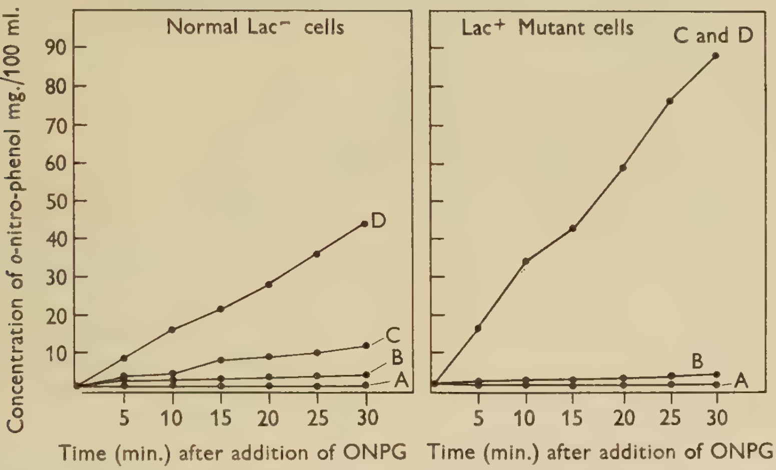

Fig. 1. β-D-Galactosidase activity of the normal lac^- organisms and lac^+ mutants of culture E.C. 8. A = organisms grown on 1 % glucose peptone agar; B = organisms grown on peptone agar; C = organisms grown on 1 % lactose peptone agar; D = organisms grown on 5 % lactose peptone agar.

The formulation of phenotypes to be expected among the paracolon bacilli

If rapid permeation of lactose to the β-galactosidase site is controlled by a specific genetically distinct permease (Cohen & Monod, 1957) one would expect a definite number of phenotypes among the naturally occurring paracolon bacilli. These phenotypes should be readily identified by their: (1) fermentative reactions towards 1 and 5 % lactose, (2) β-galactosidase activity, (3) production of lac^+ mutants possessing functional permease and galactosidase systems. The determination of hydrogenlyase activity in glucose peptone water would also distinguish between the aerogenic and anaerogenic types. Table 3 shows a tentative scheme for grouping these expected types. Normal and anaerogenic types of *Escherichia coli* have been selected to represent the naturally occurring strains of the phenotypes hydrogenlyase + (H^+), β-galactosidase + (G^+), permease + (P^+), and $H^-G^+P^+$, respectively. Cohn & Horibata (1959*a*, *b*, *c*) used the letters Y and Z to denote the galactosidase permease and β-galactosidase systems. To test the validity of such a scheme, a number of naturally occurring paracolon bacilli were examined. The majority of the strains were isolated from human faeces (normal and diarrhoeal specimens) and from water samples examined at this laboratory. All strains were biochemically or serologically distinct from the principal classified members of the Enterobacteriaceae.

Strains of *Escherichia coli*, *E. coli* anaerogenic type, *Shigella sonnei*, *S. dispar* and *S. alkalescens* were also included in this series. Approximately 10^8 organisms of each culture grown on peptone agar were introduced into 5 ml. of standard 1 % lactose and 1 % glucose peptone water containing Andrade's indicator and a Durham tube for the detection of gas. A similar inoculum was added to 5 ml. of 5 % lactose 0·1 % peptone water which was prepared according to the formula of Lowe & Evans (1957). The presence of lac^+ mutants was confirmed by plating from the fermented 1 % lactose peptone water tubes on to 1 % lactose 0·2 % peptone agar containing neutral red; lactose-fermenting colonies were confirmed by their ability to ferment 1 % lactose peptone water within 24 hr. The β-galactosidase activity was determined qualitatively by adding equal volumes (2 ml.) of 0·01 M-ONPG to dense suspensions of benzene-treated organisms which had previously been grown on peptone agar and peptone agar containing 1 % and 5 % lactose (as previously described). The mixtures were incubated at 37° for 2 hr. before reading. The presence of galactosidase activity was indicated by the originally colourless solution becoming yellow; the change was usually progressive but the final intensity varied from strain to strain.

Table 3. *A possible scheme for paracolon phenotypes*

1 % glucose peptone water fermentation	1 % lactose peptone water		5 % lactose 0·1 % peptone water fermentation	Galactosidase activity	Phenotype	Organism
	fermentation	presence of lactose-fermenting mutants				
AG	AG		A	+	$H^+ G^+ P^+$	*Escherichia coli*
AG	(AG)	+	A	+	$H^+ G^+ P^-$	Aerogenic late-lactose-fermenting Paracolon
AG	(AG)	+	(A)	–	$H^+ G^- P^+$	Aerogenic late-lactose-fermenting Paracolon*
AG	–	–	–	–	$H^+ G^- P^-$	Aerogenic non-lactose-fermenting Paracolon
A	A		A	+	$H^- G^+ P^+$	Anaerogenic *Escherichia coli*
A	(A)	+	A	+	$H^- G^+ P^-$	Anaerogenic late-lactose-fermenting Paracolon
A	(A)	+	(A)	–	$H^- G^- P^+$	Anaerogenic late-lactose-fermenting Paracolon
A	–	–	–	–	$H^- G^- P^-$	Anaerogenic non-lactose-fermenting Paracolon

AG = Acid with detectable gas within 24 hr. incubation; (AG) = acid with detectable gas after more than 24 hr. incubation; A = acid within 24 hr. incubation without detectable gas in 21 days; (A) = acid after more than 24 hr. incubation without detectable gas in 21 days. The presence of hydrogenlyase, β-galactosidase and permease is indicated respectively by the symbols H^+, G^+ and P^+.

* A mutant of this phenotype has been isolated from *E. coli* (Cohen & Monod, 1957).

The results are shown in Table 4. Among the 51 paracolon strains examined only four of the six predicted phenotypes were demonstrated. With the exception of one strain, all the aerogenic strains of the $H^+G^+P^-$ variety behaved in the predicted manner. The failure of this strain to ferment 5% lactose rapidly was also apparent with the four anaerogenic strains of the phenotype $H^-G^+P^-$, and it would appear that these strains are impermeable to 5% lactose. The strain of *Shigella sonnei* examined failed to produce detectable lac$^+$ mutants in 1% lactose peptone water even though an acid reaction was consistently obtained after incubation for 3 days; lac$^+$ mutants were, however, readily isolated from lactose-fermenting papillae which appeared on colonies growing on 1% lactose peptone agar after incubation for 3–4 days. This phenomenon was described by Cook, Knox & Tomlinson (1951). The failure to detect strains of phenotypes $H^+G^-P^+$ and $H^-G^-P^+$ might have arisen from the comparatively small numbers of strains examined.

Table 4. *The distribution of phenotypes among* 51 *naturally occurring paracolon strains*

	1% glucose peptone water	1% lactose peptone water	Detection of lac$^+$ mutants	5% lactose peptone water	Galactosidase activity	Phenotype
Paracolon strains aerogenic type						
39 strains	AG	(AG)	+	A	+	$H^+G^+P^-$
1 strain	AG	(AG)	+	(A)	+	
3 strains	AG	–	–	–	–	$H^+G^-P^-$
Paracolon strains anaerogenic type						
4 strains	A	(A)	+	(A)	+	$H^-G^+P^-$
5 strains	A	–	–	–	–	$H^-G^-P^-$
Escherichia coli	AG	AG		A	+	$H^+G^+P^+$
E. coli anaerogenic type	A	A		A	+	$H^-G^+P^+$
Shigella sonnei	A	(A)	+	(A)	+	$H^-G^+P^-$
S. dispar	A	(A)	+	(A)	+	$H^-G^+P^-$
S. alkalescens	A	–	–	–	–	$H^-G^-P^-$

AG = acid with detectable gas within 24 hr. incubation; (AG) = acid with detectable gas after more than 24 hr. incubation; A = acid within 24 hr. incubation without detectable gas in 21 days; (A) = acid after more than 24 hr. incubation without detectable gas in 21 days; –, in the fermentation reactions (columns 2 and 4) = no change in 21 days.

DISCUSSION

The segregation and phenotypic arrangement of the factors controlling fermentation of lactose afford a system whereby accurate relationships can be established between the coliform bacilli and the various types of so-called paracolon bacilli. However, before such a scheme can be presented some limitation must be placed on the terms 'coliform' ('coli-aerogenes'), 'paracolon' and 'lactose-fermentation'. It is suggested that the term 'coliform' be applied to those members of the group which produce acid with detectable gas within 24 hr. when a moderate inoculum (10^6 organisms) is incubated aerobically at 37° in 1% lactose 1% peptone water. The term 'anaerogenic coliform' could describe those strains which produce acid only within 24 hr. In the same way the terms 'paracolon' and 'anaerogenic paracolon' should be applied to those strains which produce acid with and without detectable gas after more than 24 hr., the fermentation reaction coinciding with the appearance of detectable lactose-fermenting mutants. These fermentation reactions should also be obtained under anaerobic conditions.

Table 5. *Suggested classification of coliform and paracolon bacilli*

	Coliform bacilli		Paracolon bacilli		Non-lactose-fermenting heterogeneous strains	
	Aerogenic type	Anaerogenic type	Aerogenic type	Anaerogenic type	Aerogenic type	Anaerogenic type
1 % Glucose peptone water	AG	A	AG	A	AG	A
1 % Lactose peptone water	AG	A	(AG)	(A)	–*	–*
Galactosidase activity	+	+	+	+	–	–
Detection of Lac$^+$ mutants			+	+	–	–
Phenotype	$H^+ G^+ P^+$	$H^- G^+ P^+$	$H^+ G^+ P^-$†	$H^- G^+ P^-$†	$H^+ G^- P^-$	$H^- G^- P^-$

AG = acid with detectable gas within 24 hr. incubation; (AG) = acid with detectable gas after more tha 24 hr. incubation; A = acid within 24 hr. incubation without detectable gas in 21 days; (A) = acid after mor than 24 hr. incubation without detectable gas in 21 days.

* – In the fermentation reactions = no change in 21 days.

† Galactosidase negative, permease positive strains may exist.

Table 5 sets out the distinguishing features of the coliform, the late-lactose-fermenting and the non-lactose-fermenting paracolon bacilli. It is evident that the lactose-fermenting paracolon and the coliform strains are closely related; both possess β-galactosidase and are, therefore, indistinguishable by this criterion. The non-lactose-fermenting strains, on the other hand, lack β-galactosidase and are thereby clearly distinguishable. These resemble the coliform group no more closely than other non-lactose-fermenting groups within the Enterobacteriaceae and, it could be argued, hardly merit the term 'paracolon'. If, therefore, fermentation of lactose be accepted as a fundamental characteristic of the coliform group it should also apply to the paracolon group, and some other name should be found for the non-lactose-fermenters which have hitherto been classed within the paracolon group. Alternatively, the lactose-fermenting paracolon organisms (but not the non-lactose-fermenting strains) could be included within the coliform group.

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Disaggregation of Bacterial Cell Walls by Anionic Detergents

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SUMMARY

Isolated cell walls of six Gram-negative bacteria were disaggregated by sodium dodecyl sulphate at all pH values between pH 2 and 9. Disaggregation as measured by decrease in turbidity showed maxima at pH 3 and 8. *Salmonella gallinarum* walls were disaggregated by alcohol sulphate compounds with alkyl chain lengths of 10, 12, 14 and 16 carbon atoms; maximum activity was observed with the C_{14} homologue. No sulphydryl groups were detected on disaggregation of the walls of *S. gallinarum* and *p*-chloromercuribenzoate had no protective action. Autolytic enzymes of *Vibrio metchnikovii* do not contribute to the dissolution of the walls of this organism by sodium dodecyl sulphate.

INTRODUCTION

The powerful haemolytic properties of sodium dodecyl sulphate (SDS) have long been known, and the mechanism of lysis has been explained in terms of the 'collapse' of oriented lipid and cholesterol layers in the red cell membrane (Schulman, Pethica, Few & Salton, 1955). Although Gram-negative bacteria are generally resistant to the bactericidal action of certain classes of surface-active anionic compounds (Baker, Harrison & Miller, 1941 *a*), there have been reports of killing by high concentrations (0·2 %) of SDS (e.g. Lominski & Lendrum, 1942). As the walls of Gram-negative bacteria contain appreciable amounts of lipid (Salton, 1953), it appeared likely that a direct physico-chemical disaggregation of the wall structure may occur on exposure to such surface active compounds as SDS. Salton (1957) reported the complete disaggregation of the isolated walls of several Gram-negative bacteria by SDS. This action on the surface structure of *Escherichia coli* was confirmed by Bolle & Kellenberger (1958).

METHODS

Preparation of cell walls. The cell walls of *Escherichia coli*, *Pasteurella pseudotuberculosis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella gallinarum* and *Vibrio metchnikovii* were prepared as previously described (Salton & Horne, 1951), using cells harvested from nutrient agar or nutrient broth in the stationary phase of growth. All cell-wall preparations were treated with trypsin (1 mg./ml., pH 8, 2 hr. at 37°), washed thoroughly and stored as thick suspensions in distilled water at 4°.

Disaggregation and turbidity decrease. For comparative purposes, the course and extent of 'disaggregation' of the walls by anionic surface-active compounds was followed by measuring the change in scattering of the cells walls at a wavelength of 700 mμ in a Unicam spectrophotometer (Model SP 600). That the turbidity decrease represented a disaggregation of the wall structure was strongly supported by the observation of small particles (*c.* 100 Å. diam.) in the electron microscope, and no residual thinner cell-wall substructure, under conditions of *c.* 95% turbidity decrease. The % turbidity decrease was determined from

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$$\left(\frac{\text{OD initial} - \text{OD final}}{\text{OD final}}\right) \times 100,$$

where OD = optical density measured as above.

The influence of pH value (buffers of constant ionic strength, $\mu = 0{\cdot}1$, described by Miller & Golder, 1950), temperature and alkyl chain length was investigated by following the course and extent of disaggregation by measurement of the changes in turbidity of cell-wall suspensions.

RESULTS

Influence of concentration of detergent, pH *value and temperature on the disaggregation of walls by sodium dodecyl sulphate*

The course of dissolution of walls of *Salmonella gallinarum* by various concentrations of SDS in buffer at pH 3 is shown in Fig. 1. The effect of concentration on the disaggregation of *S. gallinarum* walls as indicated by change in % turbidity was studied at pH 3, 7 and 8. The results are summarized in Fig. 2. The dissolution of the walls of various Gram-negative bacteria by 0·2% (w/v) SDS was studied at various pH values. The results are compared with those for *Salmonella gallinarum* in Table 1. The influence of temperature on the disaggregation of the walls by SDS was investigated at three temperatures, namely 18°, 37° and 53°; the results are presented in Table 2.

Table 1. *Dissolution of the walls of some Gram-negative bacteria by sodium dodecyl sulphate*

The suspensions of cell walls were adjusted to various pH values; the SDS concentration was 0·2% (w/v); incubation at 37° for 30 min.

	pH value							
	2·0	3·0	4·0	5·0	6·0	7·0	8·0	9·0
Organisms	% Turbidity decrease							
S. gallinarum	85·6	93·4	91·2	87·2	83·4	80·6	92·6	87·4
V. metchnikovii	90·2	94·4	93·2	89·8	86·6	83·5	93·7	92·1
P. vulgaris	90·5	94·6	93·5	90·2	87·2	84·4	92·4	90·3
E. coli	74·6	83·2	80·0	76·6	73·2	70·6	79·7	78·2
Past. pseudo-tuberculosis	90·1	94·2	93·2	89·8	86·6	83·4	92·0	90·4
Pseud. aeruginosa	80·2	85·2	82·4	77·2	74·4	70·6	84·4	81·8

The relationship of the alkyl chain length of anionic surface-active alcohol sulphates to their lytic activity

Concentrations of sodium decyl sulphate (C_{10}), sodium dodecyl sulphate (C_{12}), sodium tetradecyl sulphate (C_{14}) and sodium hexadecyl sulphate (C_{16}) ranging from 0·006 to 0·8% (w/v) were tested on the isolated walls of *Salmonella gallinarum* at pH 7 and 37°; the changes in turbidity are plotted in Fig. 3.

Table 2. *The effect of temperature on the dissolution of some Gram-negative walls*

Suspensions at pH 3; SDS at 0·2 % (w/v); incubated at 37° for 30 min.

	Temperature		
	18°	37°	53°
Organisms	% Turbidity decrease		
S. gallinarum	82·5*	93·4	95·5
V. metchnikovii	85·1	94·4	96·8
P. vulgaris	87·5	94·6	97·1
E. coli	70·7	83·2	83·7
Past. pseudotuberculosis	84·1	94·2	95·1
Pseud. aeruginosa	78·4	85·2	86·2

* The values presented in Table 2 represent the changes in turbidity after adjustment for any changes in the control (i.e. no SDS) cell-wall suspensions incubated at the different temperatures.

Effects of various treatments of the walls on subsequent lytic action of sodium dodecyl sulphate

Phospholipids have been shown to protect bacteria from the germicidal action of cationic and anionic detergents (Baker, Harrison & Miller, 1941*b*). The effect of lecithin on the dissolution of *Salmonella gallinarum* walls by SDS was investigated by dividing a wall suspension to which lecithin had been added into two samples, one sample was washed several times with the buffer and resuspended in buffer, the other sample being untreated. SDS was then added to both suspensions and the % turbidity decrease determined. The lecithin-treated suspension showed a turbidity decrease of 58 % as compared with an 80 % decrease for both the control (no exposure to lecithin) and the lecithin-treated and washed preparation. The results

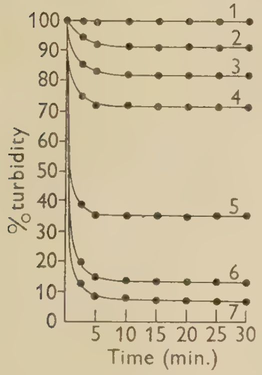

Fig. 1

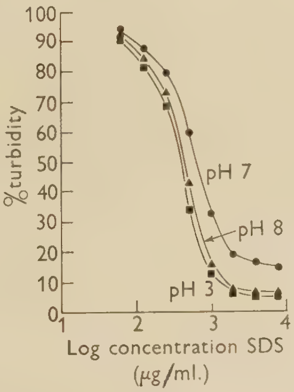

Fig. 2

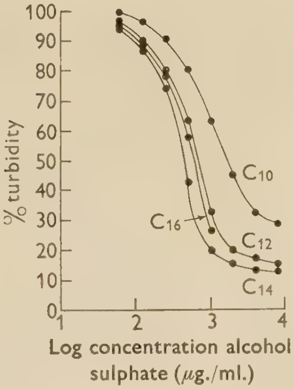

Fig. 3

Fig. 1. Disaggregation of the walls of *Salmonella gallinarum* measured by changes in turbidity at pH 3; 37°. Sodium dodecyl sulphate added to give final concentrations of: 0·006 % (curve 2); 0·012 % (curve 3); 0·025 % (curve 4); 0·05 % (curve 5); 0·1 % (curve 6) and 0·2 % (curve 7); untreated walls curve 1.

Fig. 2. Dissolution of *Salmonella gallinarum* walls by SDS at pH 3, 7 and 8; 37°.

Fig. 3. The influence of chain length of alcohol sulphates on the dissolution of walls of *Salmonella gallinarum* at pH 7; 37°.

suggest that under these conditions the 'protective' effect of lecithin was due merely to its admixture in the wall suspension rather than to strong adsorption on the wall surface and protection of vulnerable groups. If the lecithin is adsorbed, it is evident that it can be readily washed from the wall surface.

Sulphydryl groups are liberated from proteins on treatment with surface active agents (Anson, 1939), and it seemed conceivable that the disaggregating action of SDS might be due either to protein denaturation or to disruption of lipoprotein complexes in the wall. No liberation of sulphydryl groups was detected in the SDS-treated wall suspensions of *Salmonella gallinarum* under conditions which gave a detectable liberation of sulphydryl groups from bovine serum albumin, as tested by the methods of Anson (1939). These results make it appear unlikely that the disaggregation of the walls by long-chain alcohol sulphates, such as SDS, is due to denaturation of protein by disruption of –S–S– bonds. This conclusion is further strengthened by the lack of protective action of *p*-chloromercuribenzoate (concentrations 1–10 μg./ ml.) on the disaggregation of the walls of *S. gallinarum* by SDS.

It may be argued that autolytic enzymes contribute to the lysis or dissolution of the walls. That this is extremely unlikely is supported by experiments with walls of the organism *Vibrio metchnikovii* which we know to possess an active 'autolysin' which releases the mucopeptide part of the wall (Salton & Shafa, 1958). No differences in the % turbidity decrease brought about by SDS were observed as between untreated walls, walls heated for 10 min. at 100°, walls treated with 10% (v/v) formaldehyde for 3 hr. at 37°, and heated walls treated with an autolysate from *V. metchnikovii*.

DISCUSSION

In accord with earlier observations (Salton, 1957) the results of this study show that bacterial cell walls are completely disaggregated by anionic surface-active compounds such as SDS, providing the appropriate ratio of compound to wall is used. Our observations suggest that the mechanism of disaggregation of the isolated walls of *Salmonella gallinarum* (and probably other Gram-negative organisms) involves an interaction between sodium dodecyl sulphate and the lipid, lipoprotein and lipopolysaccharide part of the wall, rather than an action involving rupture of –S–S– bonds. Treatments of the walls of *S. gallinarum* does not appear to 'deterge' or remove the lipoprotein complex from the wall, leaving a residual layer of muco-complex or mucopeptide. The complete disaggregation was contrary to what we anticipated and the results of our experiments, at least in this respect, differ from what is implied from the work of Bolle & Kellenberger (1958). Our investigations suggest that the muco-complex part of the wall does not form a continuous, separate layer of the wall, but rather a reinforcing network extending across a multilayered wall (as shown in the thin sections of *Escherichia coli* by Kellenberger & Ryter, 1958).

Although anionic compounds such as SDS possess the capacity for direct disaggregation of the isolated cell walls of certain Gram-negative bacteria, the reports of inhibition of lysis by active metabolism (Bolle & Kellenberger, 1958; discussed by Newton, 1958) point to a more complicated mode of action against intact cells. If the effects can be duplicated, then it would appear that the actively metabolizing cell can exclude a compound such as SDS from its site of action on the cell wall.

It is of some interest to note the 'double maxima' effects of pH value on the disaggregation of the isolated walls. Such results are reminiscent of the data obtained for the influence of pH value on the bactericidal action of cationic compounds on cells of several Gram-negative bacteria (Salton, 1950). The explanation in terms of the cell-wall groups affected is as yet unknown.

One of the aims of the present investigation was to obtain a method for breaking the walls of Gram-negative bacteria into subunits and to obtain some idea of the size of the molecular aggregates and the possible number of different antigenic entities. Considerable difficulty in removing the SDS has been experienced and this method may therefore be unsuitable. However, it is of some interest that the disaggregated wall preparations give several lines of precipitation in agar diffusion plates with cell-wall and intact-cell antisera for *Salmonella gallinarum*.

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Excretion of Nicotinic Acid by Biotin-Deficient *Saccharomyces cerevisiae*

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SUMMARY

Growth of a strain of *Saccharomyces cerevisiae* in a glucose salts vitamins medium, containing suboptimal concentrations of biotin, was accompanied by the appearance in the medium of a free form of nicotinic acid, and also a heat-labile combined form of this substance; these were identified chromatographically as nicotinic acid and desamido-DPN (nicotinic acid adenine dinucleotide), respectively. The effect of biotin concentration and incubation time on the excretion of these substances is reported. The presence of adenine in the growth medium caused a marked decrease in the excretion of both forms of nicotinic acid, but hypoxanthine and 4-amino-5-imidazole carboxamide had no observable effect. Addition of DL-aspartic acid to the medium also brought about a decrease in the amounts excreted, although concentrations of this amino acid in excess of $0 \cdot 5 \times 10^{-3}$M stimulated excretion of desamido-DPN. It is concluded that excretion of free nicotinic and desamido-DPN is largely the result of an impairment in the ability of the biotin-deficient yeast to produce adenine, which in turn leads to a derangement in the synthesis of pyridine nucleotides.

INTRODUCTION

Several workers have studied the growth of biotin-requiring micro-organisms in media containing suboptimal concentrations of the vitamin, in order to obtain an insight into the role of biotin in the metabolism of living cells. Micro-organisms grown under these conditions have been shown to lose the ability to carry out certain metabolic reactions, principally those involving carboxylation (Lardy, Potter & Elvehjem, 1947; Shive & Rogers, 1947; Lichstein & Umbreit, 1947) and transamination (Lichstein & Christman, 1948). Biotin has also been shown to be essential for hexokinase activity in *Saccharomyces cerevisiae* (Strauss & Moat, 1958) and for succinic acid dehydrogenase activity in *Escherichia coli* (Ajl, Hart & Werkman, 1950); in *Streptococcus lactis* it is associated with ability to convert ornithine into citrulline (Ravel, Grona, Humphreys & Shive, 1959). There is evidence too of a relationship between biotin and fatty acid synthesis in lactic acid bacteria, although the nature of the relationship has not been elucidated (Hofmann, O'Leary, Yoho & Liu, 1959). Several studies have also been made on the accumulation of biosynthetic intermediates in the culture medium during growth of micro-organisms under conditions of biotin deficiency. Among the intermediates which have been detected are an aromatic amine, probably 5-amino-imidazole riboside (Chamberlain, Cutts & Rainbow, 1952; Lones, Rainbow & Woodward, 1958; Moat, Wilkins &

Friedman, 1956) and hypoxanthine (Chamberlain & Rainbow, 1954), excretion of both these compounds being the result of an impairment in the ability of biotin-deficient micro-organisms to synthesize purines. It has also been reported that under conditions of biotin deficiency, pyruvate, dimethyl-pyruvate and α-keto-glutarate are excreted by *Piricularia oryzae* (Katsuki, 1959*a*), while pyruvate can also be detected in filtrates from biotin-deficient cultures of *Bacillus macerans* (Katsuki, 1959*b*).

In a previous paper (Rose & Nickerson, 1956), growth of several biotin-requiring yeasts, in media containing suboptimal concentrations of biotin, was reported to initiate or to enhance greatly the excretion of nicotinic acid by these organisms. However, the factors which affected the excretion were not examined in detail, nor was the form in which the vitamin appeared in the growth medium ascertained. Further studies on excretion of nicotinic acid by a strain of *Saccharomyces cerevisiae* have recently been carried out in this laboratory, and this paper reports the results obtained, certain of which have already been described in brief (Rose, 1960). In light of these results, it would appear that excretion of nicotinic acid, in both free and combined forms, is largely a reflexion of the inability of the biotin-deficient yeast to synthesize adenine.

METHODS

Experimental cultures

Organism. The biotin-requiring strain of *Saccharomyces cerevisiae* (Fleischmann) used in this study was obtained from the Division of Applied Biology, National Research Council of Canada, Ottawa. It was maintained on slopes of malt wort agar (10 %, (w/v), 'Muntona' spray-dried malt extract (Munton and Fison Ltd., Stowmarket, Suffolk) + 2 % (w/v) agar); the cultures were stored at 2–4°.

Medium. The basal medium (pH 4·5) used was that described by Rose & Nickerson (1956), but with biotin omitted. Various test substances were incorporated in the medium as described later. These substances were screened for possible contamination with biotin by examining their ability to stimulate growth of the biotin-requiring strain of *Saccharomyces cerevisiae* in biotin-free medium. Substances so contaminated were washed repeatedly with ice-cold water and with 95 % (v/v) ethanol in water until they were shown to be free from detectable amounts of biotin.

Tube tests. Cultures (6 ml.) of the yeast were grown in Samco tubes as described by Northam & Norris (1951). When large volumes of culture filtrate were required, 100 ml. cultures in 350 ml. conical flasks were grown. Inocula were prepared by transferring a pin-head of growth from a 48 hr. slope culture into 6 ml. of biotin-free basal medium and incubating the culture at 25° for 18 hr., during which time the amount of organism in the culture increased from 0·09–0·11 to 0·32–0·38 mg. dry wt. yeast/ml. Organisms from these cultures were washed three times with 6 ml. portions of M/15 KH_2PO_4, and 0·6 ml. of the final suspension (6 ml.) was added to a further 6 ml. of buffer. One drop of the diluted suspension was then added to each tube containing 6 ml. biotin-free medium; flasks containing 100 ml. medium were inoculated with 17 drops of the diluted suspension. This procedure was preferred to that used previously (Rose & Nickerson, 1956) because lower blank readings were recorded when the yeast was inoculated into biotin-free basal medium. Experimental cultures were incubated statically at 25°. Growth was measured turbidimetrically, using the EEL nephelometer (model 'A') or the Hilger 'Spekker' absorptiometer (model H 760 with neutral green-grey H 508 filters), turbidity readings being related to dry weight of yeast by means of calibration curves.

Culture filtrates were obtained by centrifuging experimental cultures at 2500 rev./min., and decanting the supernatant liquid. The filtrate was adjusted to pH 4·5 with 2% (w/v) sodium hydroxide, and then sterilized by Seitz filtration ('Carlson' Grade 'E.K.' 3·5 cm. filter pads) the first 2 ml. of filtrate being rejected. Seitz filtration did not appear to alter significantly the concentrations of nicotinic acid, nicotinamide or diphosphopyridine nucleotide (DPN) in the filtrates, since excellent recovery values were obtained when standard solutions of these compounds were assayed after passage through the Seitz filter. Sterile culture filtrates were stored at 2–4° before examination.

Analytical methods

Nicotinic acid assays. The nicotinic acid contents of culture filtrates were determined microbiologically using a strain of *Candida pseudotropicalis* (National Collection of Yeast Cultures 143, Brewing Industry Research Foundation, Nutfield, Surrey), and using a modification of the method described by Williams (1946). Portions (6 ml.) of basal medium (Rose & Nickerson, 1956), containing known amounts of nicotinic acid or of sterile yeast culture filtrate, were autoclaved momentarily at 10 lb./sq.in. in Samco tubes. Inocula were prepared as described previously (Rose & Nickerson, 1956), and the cultures incubated statically at 25° for 72 hr., after which the turbidities of the cultures were measured in the EEL nephelometer. A linear relationship was obtained by plotting response (turbidity reading) against the logarithm of the dose of nicotinic acid; the assay range 20–100 mμg./tube was used in calculating the nicotinic acid contents of culture filtrates. The assay method was found to be reliable, and to give consistently valid results (Bliss, 1956).

Chromatography. Culture filtrates were examined chromatographically on Whatman no. 1 paper, using a solvent consisting of a mixture of 7 parts of 95% (v/v) ethanol in water and 3 parts M ammonium acetate, adjusted to pH 5·0 with HCl (Preiss & Handler, 1958*a*). Filtrates or extracts from filtrates were applied as spots (*c.* 3 μl.), larger amounts being applied by successive application of 3 μl. spots. Papers were irrigated for 16–20 hr. at room temperature and, when the solvent front had descended 40–42 cm., were dried with a hair dryer. Chromatogram papers were examined under ultraviolet (u.v.) radiation (Hanovia 'Chromatolite'), nicotinic acid, nicotinamide, and pyridine nucleotides then appearing as quench spots. Nicotinamide nucleotides were distinguished from the nicotinic acid analogues by placing the chromatogram paper in a tank containing a mixture of equal volumes of methyl ethyl ketone and conc. ammonia (sp.gr. 0·88) for 1 hr., after which only nicotinamide derivatives appeared as fluorescent spots under u.v. irradiation (Kodicek & Reddi, 1951). Spots of nicotinic acid and nicotinamide were further distinguished by placing the chromatogram paper in a tank over cyanogen bromide (Wang & Kodicek, 1943) for 1 hr., and then spraying with 2% (w/v) *p*-aminobenzoic acid; spots of nicotinic acid then appeared yellow and those of nicotinamide orange-yellow (Kodicek & Reddi, 1951).

RESULTS

Influence of biotin concentration and incubation time on excretion of nicotinic acid

In a series of preliminary experiments, cultures of the yeast grown in basal media supplemented with a wide range of biotin concentrations were removed at intervals, and the culture filtrates analysed for nicotinic acid. When the biotin concentration in the medium was less than $9 \cdot 0 \times 10^{-10}$ M, growth was restricted, but it was not until the concentration was below $5 \cdot 0 \times 10^{-10}$ M that nicotinic acid could be detected in the culture filtrate. Nicotinic acid activity reached a maximum in filtrates from 7-day cultures of the yeast in media containing $0 \cdot 3$–$0 \cdot 5 \times 10^{-10}$ M-biotin. Organisms from these cultures were coloured pink, but in the absence of added methionine, only very small amounts of aromatic amine were detectable in the culture filtrates (Chamberlain & Rainbow, 1954).

When filtrates from cultures of the yeast grown in media containing $0 \cdot 4 \times 10^{-10}$ M-biotin were assayed for nicotinic acid activity by adding portions of sterile filtrate aseptically to assay medium, instead of autoclaving the sample with medium, nicotinic acid potency appeared to be lower. An examination of the specificity of response of the strain of *Candida pseudotropicalis* used in the microbiological assay showed that the organism responded identically to nicotinic acid and nicotinamide, but that diphosphopyridine nucleotide (DPN) elicited a detectable growth response from the organism only in relatively high concentrations (greater than 100 mμg. nicotinic acid equivalent/ml.). It was concluded, therefore, that nicotinic acid was present in culture filtrates in a free assayable form (possibly nicotinic acid or nicotinamide), and as one or more heat-labile combined forms, which were broken down to assayable forms of the vitamin when filtrates were autoclaved momentarily at 10 lb./sq.in. with basal medium. Autoclaving for longer periods did not lead to any further increase in the nicotinic acid potency of filtrates.

Filtrates from cultures of the yeast grown in basal media containing different concentrations of biotin were therefore assayed for total nicotinic acid by using the standard assay procedure, and also for free nicotinic acid by adding portions aseptically to assay medium; the content of heat-labile, combined nicotinic acid was obtained by difference. The data in Fig. 1 show the effect of biotin concentration on growth and excretion of free and combined nicotinic acid by the yeast after incubation for 170 hr. In filtrates from media containing $0 \cdot 3$–$0 \cdot 5 \times 10^{-10}$ M-biotin, in which total nicotinic acid activity was greatest, there were approximately equal amounts of free and combined vitamin; this proportion remained roughly constant as the total amount of nicotinic acid excreted decreased with increasing biotin concentration. Nicotinic acid was not detected in filtrates from media containing more than $5 \cdot 0 \times 10^{-10}$ M-biotin. The small amount of nicotinic acid excreted in biotin-free medium was predominantly in the free form. As shown in Fig. 2, the concentrations of free and combined nicotinic acid in filtrates from cultures of the yeast grown in media containing $0 \cdot 4 \times 10^{-10}$ M-biotin increased steadily with time, reaching a maximum after about 170 hr. of incubation. Thereafter, the concentration of free nicotinic acid continued to increase, while that of the combined form decreased sharply, possibly as a result of its breakdown by enzymes liberated from the yeast cells.

Characterization of the free and combined forms of nicotinic acid

When filtrates from cultures of the yeast grown in media containing $0\cdot4 \times 10^{-10}$ M-biotin were examined chromatographically, scanning the chromatograms under u.v. irradiation revealed the presence in these filtrates of free nicotinic acid but not nicotinamide. These findings were corroborated when the papers were treated with cyanogen bromide and then sprayed with *p*-aminobenzoic acid. Similar results were obtained on examining other yeast culture filtrates which had been shown by microbiological assay to contain the free form of the vitamin.

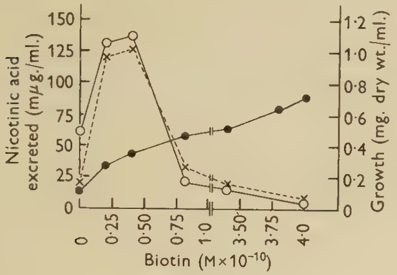

Fig. 1

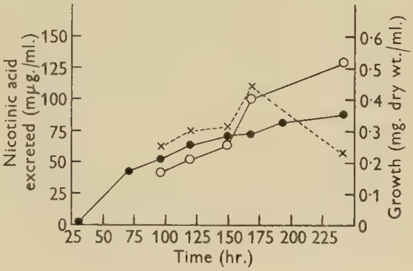

Fig. 2

Fig. 1. Effect of biotin concentration on growth (●——●) and excretion of free (○——○) and combined (×‒‒‒×) nicotinic acid by the yeast after incubation for 170 hr.

Fig. 2. Effect of incubation time on growth (●——●) and excretion of free (○——○) and combined (×‒‒‒×) nicotinic acid by the yeast in a medium containing $0\cdot4 \times 10^{-10}$ M-biotin.

When culture filtrates containing combined nicotinic acid were autoclaved momentarily at 10 lb./sq.in., and then examined chromatographically, again only nicotinic acid was detected. Since it is known that nicotinamide is not deaminated under these conditions (Preiss & Handler, 1958*a*), it was concluded that the vitamin was present in the combined form as nicotinic acid and not as the amide. The conditions required for liberation of nicotinic acid from the heat-labile combined form closely resembled those required to liberate nicotinic acid from pyridine nucleotides (Preiss & Handler, 1958*a*). However, the presence of extraneous u.v.-absorbing material precluded the possibility of examining filtrates chromatographically for the presence of pyridine nucleotides. Mercuric acetate (0·8 g. dissolved in 15 ml. water) was therefore added to portions (100 ml.) of filtrate obtained from cultures of the yeast grown in media containing $0\cdot4 \times 10^{-10}$ M-biotin, and the resulting precipitate, which included the mercuric derivatives of any pyridine nucleotides present (Singer & Kearney, 1954) was allowed to settle overnight at 2–4°. The precipitate was then decomposed by the procedure described by Chamberlain & Rainbow (1954). The extracts prepared in this way were examined chromatographically, and were shown to contain a substance having an R_F value identical with that of desamido-DPN (nicotinic acid adenine dinucleotide) an authentic specimen of which was kindly supplied by Dr N. O. Kaplan (Table 1). This spot did not fluoresce after the chromatogram paper had been treated with methyl ethyl ketone and ammonia (Kodicek & Reddi, 1951), thus confirming that it was not a nicotinamide nucleotide. No other pyridine nucleotide was detected in these extracts, although they did contain hypoxanthine (Chamberlain & Rainbow, 1954). Control chromatograms were prepared with extracts from basal media supplemented with nicotinic acid, nicotinamide, DPN, desamido-DPN, or desamido-NMN (nicotinic acid mononucleotide) which was obtained by hydrolysis of desamido-DPN (Preiss & Handler, 1958*a*). Further proof of the identity of the substance present in the extracts was obtained by hydrolysis of a portion of extract (0·5 ml.) with 0·1 N-HCl at 100° for 10 min. (Preiss & Handler, 1958*a*) when chromatographic examination of the hydrolysate revealed the presence of adenine, nicotinic acid, and of a substance having an R_F value of 0·24, in addition to some residual desamido-DPN (Table 1). A similar chromatographic pattern was obtained on examination of an extract prepared from basal medium supplemented with acid-hydrolysed desamido-DPN. No pyridine nucleotide was detected in extracts prepared from filtrates which had been autoclaved momentarily at 10 lb./sq.in.

Table 1. *R_F values of u.v.-absorbing substances in supplemented basal media and in extracts prepared from filtrates of cultures of* Saccharomyces cerevisiae

Nature of the spot	R_F value (average of three chromatograms)
Reference compounds incorporated in basal medium	
Nicotinic acid	0·76
Nicotinamide	0·79
Nicotinamide mononucleotide (NMN)*	0·31
Desamido-NMN†	0·24
Diphosphopyridine nucleotide (DPN)	0·19
Desamido-DPN	0·14
Adenine	0·65
Hypoxanthine	0·62
U.v.-absorbing spots in extracts from culture filtrates	0·15
	0·61
	0·76
U.v.-absorbing spots in extracts from culture filtrates after acid hydrolysis	0·14
	0·24
	0·61
	0·63
	0·77

* Obtained by hydrolysis of DPN.

† Obtained by hydrolysis of desamido-DPN (Preiss & Handler, 1958*a*).

Effect of adenine and of certain related substances on excretion of nicotinic acid

It is well established that biotin is concerned in the biosynthesis of purines in *Saccharomyces cerevisiae* (Chamberlain & Rainbow, 1954; Moat *et al.* 1956). However, as reported by Chamberlain & Rainbow (1954), addition of adenine to media containing suboptimal concentrations of biotin brought about only a slight stimulation of growth, although in concentrations above $0{\cdot}5 \times 10^{-3}$ M-adenine, the organisms did not have the pink colour characteristic of the biotin-deficient yeast. When media containing $0{\cdot}4 \times 10^{-10}$ M-biotin were supplemented with adenine, excretion of both free and combined nicotinic acid decreased (Fig. 3). Excretion of the free form of the vitamin was more sensitive to the presence of adenine, and at $0{\cdot}7 \times 10^{-3}$ M-adenine, the amount had dropped to less than one-tenth of that excreted in adenine-free medium; increased concentrations of adenine produced no further decrease in the amount of nicotinic acid excreted. The effect on excretion of the combined form of nicotinic acid was somewhat less marked, adenine at $3{\cdot}5 \times 10^{-3}$ M being required to produce maximum effect.

However, addition of hypoxanthine or 4-amino-5-imidazole carboxamide, ribotide derivatives of which are concerned in the biosynthesis of purines, had no observable effect, even at $4{\cdot}0 \times 10^{-3}$ M, on growth of, or nicotinic acid excretion by, the yeast when growing in media containing $0{\cdot}4 \times 10^{-10}$ M-biotin.

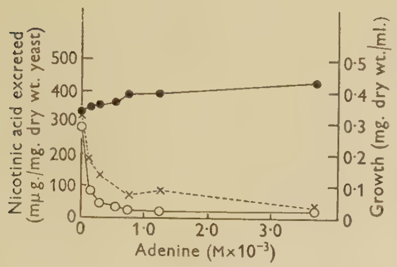

Fig. 3

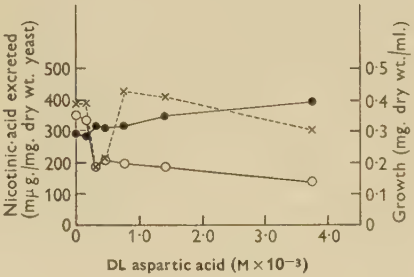

Fig. 4

Fig. 3. Effect of adenine concentration on growth (●——●) and excretion of free (○——○) and combined (×– – –×) nicotinic acid by the yeast in a medium containing $0{\cdot}4 \times 10^{-10}$ M-biotin after incubation for 181 hr.

Fig. 4. Effect of DL-aspartic acid concentration on growth (●——●) and excretion of free (○——○) and combined (×– – –×) nicotinic acid by the yeast in a medium containing $0{\cdot}4 \times 10^{-10}$ M-biotin after incubation for 167 hr.

Koser, Wright & Dorfman (1942) reported that aspartic acid was able to replace partially the biotin requirement of the yeast *Candida pseudotropicalis*; a similar effect was later reported for strains of *Saccharomyces cerevisiae* (Moat & Emmons, 1954). The data in Fig. 4 show the effect of DL-aspartic acid on growth and nicotinic acid excretion by the yeast growing in a medium containing $0{\cdot}4 \times 10^{-10}$ M-biotin. The amino acid had only a slight stimulatory effect on growth of the yeast, although at concentrations above $0{\cdot}5 \times 10^{-3}$ M, the organisms appeared creamy-white instead of pink. Concentrations of aspartic acid up to $0{\cdot}5 \times 10^{-3}$ M caused a decrease in the amounts of free and combined vitamin excreted; but whereas the addition of further aspartic acid had little effect on the amount of free nicotinic acid excreted, the concentration of desamido-DPN in culture filtrates increased sharply, and only decreased when the concentration of amino acid in the medium exceeded $2{\cdot}0 \times 10^{-3}$ M.

DISCUSSION

Accumulation of biosynthetic intermediates by micro-organisms growing under conditions of metabolic stress has been studied with the object of elucidating the intermediate stages during the biosynthesis of a cell component, and also to discover the mode of action of the metabolic stress. Nicotinic acid occurs in living cells in the form of pyridine nucleotides, DPN and TPN, and accumulation in the culture medium of nicotinic acid and desamido-DPN suggests that synthesis of pyridine nucleotides by *Saccharomyces cerevisiae* is impaired under conditions of biotin deficiency. A deficiency of pyridine nucleotides in the biotin-deficient cells was not demonstrated experimentally in the present work, although this situation has been reported with *Piricularia oryzae* (Katsuki, 1959*a*) and with *Bacillus macerans* (Katsuki, 1959*b*).

The sequence of reactions involved in the biosynthesis of pyridine nucleotides in several different types of tissue has been studied in various laboratories (Preiss & Handler, 1958*b*; Langan, Kaplan & Shuster, 1959). These studies have indicated that synthesis of DPN proceeds via nicotinic acid, nicotinic acid mononucleotide (desamido-NMN) and desamido-DPN. Accumulation of two of these intermediates namely, nicotinic acid and desamido-DPN, in biotin-deficient cultures of *Saccharomyces cerevisiae* is in agreement with the finding by Preiss & Handler (1958*b*) that this biosynthetic pathway operates in yeast. The absence of nicotinamide from yeast culture filtrates, either free or in nucleotide form, is also significant, for it gives support to the contention that this amide is formed only by degradation of nicotinamide nucleotides, and that free nicotinamide does not participate directly in the synthesis of these nucleotides (Hughes & Williamson, 1952). Also, since free nicotinic acid and a nicotinic acid nucleotide accumulate under conditions of biotin deficiency, it might be concluded that synthesis of nicotinic acid by the yeast does not require the presence of biotin. In this connexion it may be mentioned that Dalgliesh (1955) reported that in the rat conversion of tryptophan to nicotinic acid was not affected by biotin deficiency.

Accumulation of free nicotinic acid and desamido-DPN in the culture medium under conditions of biotin deficiency suggests that biotin is essential for further metabolism of these intermediates, the vitamin functioning either co-enzymically or in the synthesis of other participants in the reactions. Adenine is required for synthesis of pyridine nucleotides from nicotinic acid, and since biotin is essential for the synthesis of purines in *Saccharomyces cerevisiae* (Chamberlain & Rainbow, 1954; Moat, Wilkins & Friedman, 1956) it might be expected that, under conditions of biotin deficiency, biosynthesis of pyridine nucleotides would be deranged. The marked decrease in excretion of both forms of nicotinic acid that was observed in the present work on adding adenine to the medium suggests that a deficiency of adenine was largely responsible for the impairment in pyridine nucleotide synthesis. This conclusion is further supported by the observation that there was no significant effect on nicotinic acid excretion following addition of hypoxanthine or 4-amino-5-imidazole carboxamide, the ribotide derivatives of which are concerned in purine biosynthesis, but which require the presence of biotin for further metabolism (Moat *et al.* 1956). Adenine was reported to be required in the form of ATP for the conversion of desamido-NMN into desamido-DPN and for the amidation of desamido-DPN (Preiss & Handler, 1958*b*). Katsuki (1959*a*, *b*) has reported that *Piricularia oryzae* and *Bacillus macerans*, grown in media containing suboptimal concentrations of biotin, contained considerably decreased amounts of ATP as well as of DPN; he concluded that the decreased amounts of DPN were due, not to the absence of DPN-synthesizing enzymes, but to deficiency of ATP. From the difference in the concentrations of adenine required to bring about an equivalent decrease in the excretion of free and combined nicotinic acid by the yeast, it might be concluded that the presence of adenine in the medium more easily facilitates the further metabolism of nicotinic acid than the amidation of desamido-DPN. Since ATP is required for both transformations, it is conceivable that the reactions proceed at different sites within the cell, or alternatively, that biotin is also required *per se* in amidation of desamido-DPN or in the synthesis of glutamine which has been reported to participate in the amidation reaction.

The role of aspartic acid in purine synthesis (Wahba & Shive, 1954), and in amino group transfer in the purine skeleton (Abrams & Bentley, 1955) may well explain the biotin-sparing action of this amino acid. From Fig. 4 it can be seen that the effect of aspartic acid on growth of the yeast in biotin-deficient medium was slight, and was, in fact, less marked than previously reported for strains of *Saccharomyces cerevisiae* (Chamberlain & Rainbow, 1954; Rose & Nickerson, 1956) and of other micro-organisms growing under comparable conditions of biotin deficiency; this might, however, be a result of using biotin-deficient yeast cells in the inoculum. But although the effect of the amino acid on growth was slight, excretion of free and combined nicotinic acid was decreased by adding the amino acid up $0{\cdot}5 \times 10^{-3}$M. Addition of more aspartic acid had little further effect on excretion of the free form of nicotinic acid; on the other hand, excretion of desamido-DPN was stimulated, although at higher concentrations there was a decrease in the amount which appeared in the growth medium. An explanation of this stimulatory effect is not immediately apparent, although aspartic acid may act indirectly by stimulating synthesis of nicotinic acid. However, even in the presence of $3{\cdot}75 \times 10^{-3}$M-aspartic acid, substantial amounts of free and combined nicotinic acid were excreted, which indicates that the biotin requirement in certain of the biotin-dependent reactions concerned in purine synthesis cannot be replaced by aspartate. It is concluded, therefore, that excretion of nicotinic acid and desamido-DPN by the strain of *S. cerevisiae* used in the present work, when growing in media containing sub-optimal concentrations of biotin, is a result of the inability of the biotin-deficient yeast to produce adenine, and that this causes a derangement in pyridine nucleotide synthesis, leading to the excretion of the two biosynthetic intermediates.

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Mineral Requirements and other Characters of Selected Marine Bacteria

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SUMMARY

Marine bacteria from Atlantic coastal waters off Florida, isolated by a technique assuring that all would grow on a sea-water medium but not on a distilled water medium, were studied; 96 of 100 original isolates were characterized by morphological and some physiological properties, particularly those related to mineral responses. All isolates were Gram-negative rods or spiral forms, grew well in a simple saline-peptone medium, and most were pigmented and motile. All isolates required NaCl for good growth, and groupings could be clearly established for those whose mineral nutrition was satisfied, in a casein digest broth, by adding NaCl only, NaCl + KCl, NaCl + Mg salts, or all three salts. Lytic susceptibility in various mineral solutions and growth tolerance to various salt levels were studied. Some overall similarities, and a few correlations between mineral nutrition and physiological versatility, were established.

INTRODUCTION

Only recently has intensive investigation been initiated with marine bacteria concerning those properties which distinguish them physiologically from terrestrial bacteria. ZoBell (1946) summarized much of the research done to that date, showing that the ability to grow in and requirement for sea water as a base for media characterized these bacteria; solution mixtures of the major mineral components were shown to be substitutes for natural sea water for many isolates. Studies of mineral nutrition and osmotic relations have been reported for several marine bacteria (MacLeod, Onofrey, & Norris, 1954; Pratt, Boring, & Riley, 1954). The investigations reported here deal with the isolation and effects of major sea-water minerals on 96 stenohaline marine bacteria.

Studies with marine bacteria (ZoBell, 1946) and halophilic bacteria (Flannery, 1956) have shed relatively little light on the detailed mineral requirements of the former group; somewhat more information was available concerning the bacteria isolated from salt-preserved material (e.g. Robinson & Gibbons, 1952; Ingram, 1957). Luminescent bacteria, from marine sources, have been shown to be susceptible to lysis in particular salt concentrations (Johnson & Harvey, 1937; Johnson & Gray, 1949) and physiologically responsive to varied salts (Johnson & Harvey, 1938). However, the highly halophilic bacteria differ materially from the marine bacteria described by ZoBell & Upham (1944) and the restricted groups studied by us and by MacLeod *et al.* (1954).

Our results support the view of MacLeod & Onofrey (1956, 1957) that at least one major group of marine bacteria may be distinctly characterized by an absolute requirement for sodium ions (see also Ingram, 1957). Our studies have extended this observation to nearly 100 isolates (MacLeod *et al.*, detailed the requirement for six of 15 marine forms) and clearly demonstrated that requirements for other major sea-water minerals varied among the isolates and could be used to segregate them into groups; within these groups, a few correlations were found with other physiological properties.

METHODS

Basal media. The basal medium used for isolation, and for all routine cultural, morphological, and physiological tests consisted of Trypticase (Baltimore Biological Laboratory, Baltimore, Md., U.S.A.), a pancreatic digest of casein, 1 % (w/v) in sea water; 2 % (w/v) agar was used for solid medium. Modifications for certain physiological tests are described below. Special experiments concerning mineral requirements and salt tolerance necessitated use of media containing 0·1 % (w/v) trypticase, and an artificial sea water (A.S.W.) composed of reagent grades of (%, w/v) NaCl, 2·4 (*c.* 0·4 M); KCl, 0·07 (*c.* 0·01 M); $MgCl_2.6H_2O$, 0·53 (*c.* 0·026 M); and $MgSO_4.7H_2O$, 0·70 (*c.* 0·028 M). The reaction of all media, without adjustment, was pH 6·9 – 7·2.

Mineral requirements. Preliminary experiments, confirmed by later data, demonstrated that all of the isolates grew satisfactorily on the trypticase-A.S.W. medium; neither sea water, nor complex mixtures of trace minerals were required. Initially, test media consisted of sea water (control); artificial sea-water concentrations of NaCl, NaCl + KCl, $NaCl + MgCl_2 + MgSO_4$, $KCl + MgCl_2 + MgSO_4$, or all the minerals; and distilled water; each with 1 % (w/v) trypticase. In later tests, the trypticase was used at 0·1 % (w/v) concentration, sea-water medium was omitted, and a test medium was added in which the only salt component was NaCl (0·6 M) at the concentration of the total salt content of sea water. Inoculum was washed from an 18 to 36 hr. culture on 0·1 % trypticase-A.S.W. agar with sterile 0·4 M-NaCl solution; 0·1 ml. of the dilute suspension (adjusted to O.D. 0·30 ± 0·05) was added to 30 ml. of test medium in 250 ml. Erlenmeyer flasks. Growth in test media was estimated by reading optical density at 500 mμ in a 'Spectronic 20' spectrophotometer (Bausch and Lomb Optical Co., Rochester, New York, U.S.A.). Precautions were taken to minimize mineral contamination: solutions were prepared with distilled water, and glassware was washed with near-neutral detergent, followed by thorough rinsing with distilled water; test cultures were incubated with stainless steel caps. In A.S.W. media, the trypticase was separately sterilized and added aseptically to sterile mineral solutions to avoid precipitation.

Characterization tests. Various tests to characterize the general morphology and cultural features of the isolates were performed according to standard techniques (*Manual of Microbiological Methods*, 1957). Colonial appearance and pigmentation were observed and recorded after 24–36 hr. at 27° on basal agar medium. Morphology was determined from smears prepared by the agar block technique, fixed in Bouin's solution, and stained by Burke's modification of the Gram stain. Physiological tests were performed in basal broth or agar to which was added the test substrate when necessary, the cultures being incubated statically at 27° for 12–36 hr. Carbohydrates were separately sterilized and added aseptically to sterile broth; 0·02 % (w/v) each of L-cystine and ferric ammonium citrate were used as supplements in tests for production of H_2S and indole. Tests for gelatin and starch hydrolysis and for nitrate reduction to nitrite or N_2 were performed conventionally.

Lytic susceptibility. The tendency of the isolates to lyse in various mineral solutions was tested by the method of Pratt & Riley (1955) with minor modifications. A concentrated suspension of test bacteria, subsequently adjusted to give an optical density of *c.* 0·80 at 500 mμ in a Coleman Junior spectrophotometer (Coleman Instruments, Inc., Maywood, Illinois, U.S.A.) when diluted 1:100 in sea water, was harvested with sea water from basal agar plate cultures; with a few agar-digesting isolates, the cells were centrifuged from shaken, broth cultures and resuspended in sea water. The adjusted suspension was added, 0·1 ml. to 10 ml. of each of the test solutions in optically matched tubes, dispersed by inversion, and the tubes were incubated in a water bath at 37° for 15 min. The optical density of each suspension and of a control suspension in sea water was then read in the spectrophotometer, the residual turbidity of tests being referred to the control as 100 %. Test solutions were distilled water and 0·50 M and 0·05 M solutions each of NaCl, KCl, and $MgCl_2$.

Salt tolerance. The ability of 15 selected isolates to multiply in salt concentrations different from that in sea water was tested in shaken broth cultures in 0·1 % (w/v) trypticase-A.S.W., with NaCl-concentrations of 0·2, 0·4, 0·8, 1·4, 2·0 and 2·6 M, the other minerals being added at A.S.W. levels. Inocula were prepared and used as described for the mineral requirement tests. The shaken cultures, incubated at 30°–32°, were observed at 24 and 48 hr., relative growth being recorded according to visual judgment.

RESULTS

Isolation of cultures. A sample of water was taken several yards offshore on Florida's Atlantic coast at Matanzas Beach and another at Crescent Beach, distant from the mouth of any freshwater stream. The water was transported immediately to the laboratory and 0·1 ml. samples, six from the Matanzas Beach water and four from the Crescent Beach water, were spread on the surface of basal agar plates. When colonies developed, after incubation at 27° for 24–36 hr., replica plates (Lederberg & Lederberg, 1952) were made on basal (sea water) agar and on distilled water-trypticase agar. Isolates were selected from the replica plates after incubation, with choice being made from sea-water plates of colonies which had no replicas on the distilled water medium. Of 100 isolates thus selected, numbered M.B. 1 to M.B. 100, 96 were used in this study.

General characterization. Tests of morphological and physiological properties were inadequate to classify the isolates, but sufficed to serve as pragmatic means of distinction among them. Detailed data are not reported here, but certain of the observations are summarized. A majority of the isolates (57) formed pigment, most of them producing a cream to yellow pigment which developed slowly and became pronounced after several days' incubation. One (M.B. 66) produced a bright red, water-insoluble pigment. All cultures were Gram-negative rods or, in 14 instances, vibrio to spiral forms. Only 41 fermented one or more carbohydrates, and 42 hydrolysed starch. However, 76 hydrolysed gelatin and 72 were motile. Nitrite, but no gas, was produced from nitrate by 29 isolates, 11 produced H_2S, and 14 produced indole.

Minerals required for good growth. Preliminary tests showed that the 96 isolates all grew satisfactorily in 0·1 % (w/v) trypticase broth or agar containing a mixture of the major minerals of sea water. Each was inoculated, therefore, into tubes containing sterile 0·1 % (w/v) trypticase broth containing various combinations of salts: all components of A.S.W.; NaCl and KCl; NaCl, $MgCl_2$ and $MgSO_4$; KCl, $MgCl_2$ and $MgSO_4$; and NaCl only; each salt at the concentration in sea water (see Methods). An additional test medium contained, as the sole added salt, NaCl at 0·6 M, approximating the total salt content of sea water. Since previous tests had shown that growth in media without added NaCl was nil or negligible, inocula were prepared by suspension in 0·4 M-NaCl of the growth from 18 to 36 hr. cultures on 0·1 % trypticase-A.S.W. agar; 0·1 ml. was added to 30 ml. of test medium (300-fold dilution).

Results of these tests (Tables 1–4) demonstrated the clear-cut discriminations observable with the majority of cultures; four mineral requirement groups were obvious, as had previously been suggested by Waddell (1956). Thirty-five isolates grew significantly only when all four salts were present (Table 1); most of them grew prolifically. Only one (M.B. 59) showed slight turbidity in the presence of added NaCl only. Seventeen isolates grew sufficiently to give a turbidimetric measurement when only NaCl and magnesium salts were added (Table 2); in three instances, some growth occurred when NaCl only was added at the higher concentration. Twenty-five isolates grew adequately with addition of NaCl and KCl only (Table 3), in most instances as well as or better than in the A.S.W. medium. With seven of this group, very slight growth occurred either in the sodium-magnesium medium, or in that containing NaCl only. With the remaining 19 cultures (Table 4), growth in the medium containing added NaCl only was appreciable, and generally these isolates grew in any salt combination of which NaCl was a component; two isolates failed to produce measurable turbidity in the sodium + magnesium salt or sodium + potassium salt combinations, the reason not being apparent from the experimental data. Only four of the 96 isolates grew to measurable turbidity in the medium to which no NaCl was added (K, Mg medium), and in each instance the growth was appreciably less than in any of the media containing NaCl.

Lytic susceptibility. The sensitivity of marine isolates to lowered osmotic pressure and to particular salt solutions resulting in lysis was reported by Pratt & Riley (1955) for the selected types studied in this laboratory. The quantitative variability of results among the 96 isolates in the various test solutions made interpretation difficult; the results (Tables 1–4) are recorded according to an arbitrary classification, based on maintenance of at least 90 % turbidity in test solutions as compared to the control: 1, all test solutions; 2, none; 3, 0·5 M-$MgCl_2$ only; 4, 0·5 and 0·05 M-$MgCl_2$ only; 5, 0·5 M-NaCl, 0·5 and 0·05 M-$MgCl_2$; and 6, 0·5 M-NaCl, KCl, and $MgCl_2$, and 0·05 M-$MgCl_2$.

It was evident that the majority of cultures were susceptible to loss of optical density in distilled water and in 0·1 M and 0·5 M-KCl. NaCl and $MgCl_2$ at 0·5 M, and $MgCl_2$ at 0·05 M, however, provided protection to a majority of the isolates. These data do not reveal the response of isolates in terms of viability, nor are certain other equivocal aspects evident. These are the subject of continuing investigation.

Salt tolerance. Some attempts have been made (Flannery, 1956; Ingram, 1957), without marked success, to classify marine and halophilic bacteria together (or in some instances, apart) on the basis of ability to grow or survive in various levels of salt. Consequently, 15 isolates, selected to represent the four mineral requirement groups, were studied by cultivation in shaken broth cultures, the media being

Table 1. *Growth of marine isolates requiring all major minerals of sea water*

Isolate no. M.B.	A.S.W.	O.D. in 0·1 % trypticase broth with indicated minerals added*						Lytic prop.†	Fermentations‡						Other activity				
		Na, Mg	Na, K	Mg, K	0·4 M NaCl	0·6 M NaCl	None		Glucose	Xylose	Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H_2S	Indole
4	1·00	0	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
6	1·10	0	0	0	0	0	0	3	−	−	−	−	−	−	−	+	−	−	−
8	0·95	0	0	0	0	0	0	3	−	−	+	−	−	−	−	+	−	−	−
10	0·66	0	0	0	0	0	0	5	−	−	−	−	−	−	+	+	−	−	−
11	1·30	0	0	0	0	0	0	3	−	−	−	−	−	−	−	+	−	−	−
15	1·10	0	0	0	0	0	0	3	−	−	−	−	−	−	−	+	−	−	−
17	1·10	0	0	0	0	0	0	3	−	−	−	−	−	−	+	+	−	−	−
18	0·62	0	0	0	0	0	0	5	−	−	−	−	−	−	−	+	−	−	−
19	1·10	0	0	0	0	0	0	5	−	+	−	−	−	−	−	+	−	−	−
23	0·92	0	0	0	0	0	0	6	−	−	−	−	−	−	−	+	−	−	−
26	0·80	0	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
27	0·75	0	0	0	0	0	0	6	−	−	−	−	−	−	+	+	−	−	−
29	0·91	0	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
34	0·95	0	0	0	0	0	0	5	−	−	−	−	−	−	−	+	−	−	−
35	0·76	0	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
36	1·10	0	0	0	0	0	0	2	−	−	−	−	−	−	+	+	−	−	−
42	0·86	0	0	0	0	0	0	4	−	−	−	−	−	−	−	+	−	−	−
43	0·64	0	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
44	0·97	0	0	0	0	0	0	4	−	−	−	−	−	−	−	+	−	−	−
45	1·10	0	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
51	0·27	0	0	0	0	0	0	5	−	−	−	−	−	+	−	−	−	−	−
52	0·35	0	0	0	0	0	0	3	−	−	+	−	−	−	−	+	+	−	−
59	1·00	0	0	0	0·08	0·06	0	5	−	−	−	−	−	−	−	+	−	+	−
62	1·10	0	0	0	0	0	0	5	−	−	−	−	−	−	+	+	−	−	−
64	0·55	0	0	0	0	0	0	6	−	−	−	−	−	−	+	+	−	−	−
69	1·00	0	0	0	0	0	0	5	−	−	−	−	−	−	−	+	−	−	−
77	0·76	0	0	0	0	0	0	5	−	−	−	−	−	−	+	+	+	−	−
78	0·35	0	0	0	0	0	0	5	−	−	−	−	−	−	−	−	−	−	−
87	0·31	0	0	0	0	0	0	1	−	−	−	−	−	−	−	+	+	−	−
91	0·38	0	0	0	0	0	0	5	−	−	−	−	−	−	−	+	+	−	−
92	0·48	0	0	0	0	0	0	1	−	−	−	−	−	−	−	+	−	−	−
93	0·36	0	0	0	0	0	0	3	−	−	−	−	−	−	−	+	−	−	−
94	0·17	0	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
97	0·67	0	0	0	0	0	0	2	−	−	−	−	−	−	+	+	−	−	−
98	0·90	0	0	0	0	0	0	2	−	−	−	−	−	−	+	+	−	−	−

* Measured in spectrophotometer at 500 mμ. Medium contained indicated minerals as NaCl, KCl, and $MgCl_2 + MgSO_4$. Incubated on shaker at 30° for 12–24 hr. (until maximum turbidity was reached).

† Lytic property: O.D. $\geqq$ 90 % of S.W. control in: 1, all solutions; 2, no solutions; 3, 0·5 M-$MgCl_2$ only; 4, 0·5 M and 0·05 M-$MgCl_2$ only; 5, 0·5 M-NaCl, 0·5 and 0·05 M-$MgCl_2$; 6, 0·5 M-NaCl, KCl and $MgCl_2$ and 0·05 M-$MgCl_2$.

‡ + = produced acid in statically incubated broth tubes; − = no acidity.

0·1 % (w/v) trypticase made with solutions of the minerals of artificial sea water, in which the NaCl concentration was adjusted to 0·2, 0·4, 0·8, 1·4, 2·0, or 2·6 M. Failure to grow was judged by the absence of visible turbidity in 48 hr. at 30°–32°. The data are shown in Table 5.

It was apparent that some of the isolates tested could not multiply in the medium containing 2·0 M-NaCl, and none grew in 2·6 M-NaCl medium. All 15 isolates tested

Table 2. *Growth of marine isolates requiring sodium and magnesium salts*

Isolate no. M.B.	A.S.W.	O.D. in 0·1 % trypticase broth with indicated minerals added*						Lytic prop.†	Fermentations‡						Other activity				
		Na, Mg	Na, K	Mg, K	0·4 M-NaCl	0·6 M-NaCl	None		Glucose	Xylose	Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H_2S	Indole
7	0·90	0·53	0	0	0	0	0	1	−	−	−	−	−	−	−	+	−	−	−
16	1·05	0·52	0	0	0	0	0	1	−	−	−	−	−	−	−	+	−	−	−
24	0·87	0·20§	0	0	0	0	0	5	−	+	−	−	−	−	+	+	−	−	−
31	0·30§	0·05§	0	0	0	0	0	4	−	−	−	−	−	−	+	+	−	−	−
33	1·30	0·26	0	0	0	0	0	5	−	−	−	−	−	−	−	+	−	−	−
38	0·62	0·33	0	0	0	0	0	1	−	−	−	−	−	−	−	+	−	−	−
50	1·40	0·41	0	0	0	0	0	5	−	−	−	−	−	−	−	+	−	−	−
54	0·04	0·11	0	0	0	0	0	1	−	−	+	−	−	−	−	−	−	−	−
65	1·00	0·34	0	0	0	0	0	1	−	−	−	−	−	−	−	+	−	−	−
67	0·81	0·29	0	0	0	0	0	5	−	−	−	−	−	−	+	+	−	−	−
71	0·46	0·20	0	0	0	0	0	5	−	−	−	−	−	−	−	+	−	−	−
80	0·26	0·08	0	0	0	0	0	5	−	−	−	−	−	−	−	+	−	−	−
81	0·27	0·28	0	0	0	0	0	3	+	−	−	−	−	+	−	−	−	−	−
89	0·37	0·12	0	0	0	0	0	5	−	+	−	−	−	−	+	+	−	−	−
48	0·95	0·29	0	0	0·07	0·07	0	5	−	−	−	−	−	−	−	+	−	−	−
83	1·20	0·43	0	0	0	0·20	0	5	−	−	−	−	−	−	−	+	−	−	−
84	1·00	0·35	0	0	0	0·23	0	5	−	−	−	−	−	−	−	+	−	−	−

*, †, ‡. See Table 1.

§ Growth very flaky-gelatinous, probably greater than indicated by O.D.

Table 3. *Growth of marine isolates requiring sodium and potassium salts*

Isolate no. M.B.	A.S.W.	O.D. in 0·1 % trypticase broth with indicated minerals added*						Lytic prop.†	Fermentations‡						Other activity				
		Na, Mg	Na, K	Mg, K	0·4 M-NaCl	0·6 M-NaCl	None		Glucose	Xylose	Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H_2S	Indole
1	0·83	0	0·54	0	0	0	0	3	−	−	−	−	−	−	+	+	+	−	−
3	0·77	0	0·75	0	0	0	0	5	+	−	+	−	−	+	+	−	+	−	+
5	0·85	0	0·66	0	0	0	0	3	+	−	+	−	−	−	−	−	+	+	+
9	1·20	0	0·15	0	0	0	0	5	+	−	−	−	−	−	−	−	−	+	+
12	0·80	0	0·64	0	0	0	0	3	+	−	+	−	−	+	−	−	+	−	−
14	0·76	0	0·88	0	0	0	0	6	+	−	+	−	−	+	+	+	+	−	+
20	0·78	0	0·52	0	0	0	0	5	+	−	+	−	+	−	−	+	+	−	−
22	0·72	0	0·68	0	0	0	0	5	+	−	+	−	+	+	−	−	+	+	+
25	0·47	0	1·10	0	0	0	0	5	+	−	+	−	+	+	+	−	+	−	+
28	1·40	0	1·10	0	0	0	0	3	+	−	+	−	−	−	−	−	+	+	+
39	1·20	0	0·86	0	0	0	0	5	+	−	+	−	−	−	−	−	+	−	+
40	1·10	0	0·64	0	0	0	0	5	+	−	+	−	−	−	−	−	+	−	+
58	0·11	0	0·12	0	0	0	0	2	−	−	−	−	−	−	−	−	−	−	−
61	0·17	0	0·10	0	0	0	0	2	−	−	−	−	−	−	−	−	+	−	−
76	0·37	0	0·24	0	0	0	0	5	+	+	+	−	−	−	−	+	−	−	−
82	0·10	0	0·31	0	0	0	0	6	+	−	+	−	+	−	−	−	+	+	+
90	0·23	0	0·43	0	0	0	0	3	+	−	+	−	−	−	+	+	+	−	+
95	0·11	0	0·07	0	0	0	0	5	−	−	−	−	−	−	−	−	−	−	−
30	0·41	0·04	0·42	0	0	0	0	1	+	−	+	−	−	−	+	+	−	−	−
57	0·44	0	0·16	0	0·05	0	0	5	−	−	−	−	−	−	+	+	+	−	−
60	0·58	0·16	0·58	0	0	0	0	5	+	−	+	−	−	−	+	+	+	−	−
63	1·10	0	0·66	0	0·05	0	0	5	+	−	+	−	−	−	+	+	+	−	−
68	0·75	0·13	0·24	0	0	0	0	6	−	−	−	−	−	−	−	+	−	−	−
75	0·38	0·11	0·31	0	0	0	0	5	+	−	+	−	−	−	+	+	−	−	−
86	0·40	0·11	0·25	0	0	0	0	5	−	−	+	−	−	−	−	+	−	−	−

*, †, ‡. See Table 1.

Table 4. *Growth of marine isolates requiring sodium salts only*

		O.D. in 0·1 % trypticase broth with indicated minerals added*							Fermentations‡						Other activity				
olate no. .B.	A.S.W.	Na, Mg	Na, K	Mg, K	0·4 M-NaCl	0·6 M-NaCl	None	Lytic prop.†	Glucose	Xylose	Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H_2S	Indole
2	0·95	0·75	0·43	0	0·28	0·16	0	2	+	−	−	−	−	−	+	+	−	−	−
13	0·82	0·36	0·90	0	0·38	0·17	0	6	+	−	+	−	−	−	+	+	+	+	+
21	0·90	0·38	0·95	0·15	0·41	0·38	0	6	−	−	+	−	+	+	−	+	+	+	+
32	0·51	0·13	0·56	0	0·20	0·03	0	5	+	−	+	−	+	+	+	+	+	+	−
37	0·89	0·19	1·00	0	0·15	0	0	2	−	−	+	−	−	−	−	+	+	+	−
41	1·00	0·54	0·70	0	0·26	0·25	0	4	−	−	−	−	−	−	−	+	−	−	−
47	0·87	0·42	0·98	0·19	0·48	0·40	0	3	−	+	−	−	−	−	−	−	−	−	−
49	0·70	0·23	0	0	0·29	0·19	0	5	+	−	−	−	−	−	+	+	−	−	−
53	0·95	0·33	1·00	0·14	0·35	0·34	0	5	−	−	−	−	−	−	−	+	−	−	−
55	0·90	0·32	0·64	0	0·18	0·14	0	2	+	−	−	−	−	−	+	+	−	−	−
56	0·68	0·05	0·56	0	0·10	0	0	5	−	−	+	−	−	−	−	−	−	−	−
66§	0·35	0·36	0·23	0	0·23	0·30	0	5	+	+	+	−	+	+	+	+	−	−	−
72	0·30	0·14	0	0	0·20	0	0	4	+	−	+	−	−	+	+	+	+	−	+
73	0·75	0·19	0·60	0	0·31	0·19	0	5	+	−	+	−	−	−	+	+	−	−	−
74	0·45	0	0·35	0	0·14	0	0	5	+	−	+	−	−	−	+	+	−	−	−
88	0·37	0·16	0·17	0	0·13	0·05	0	5	−	+	−	−	−	−	−	−	−	−	−
96	0·29	0	0·23	0	0·11	0	0	6	+	−	+	−	−	−	+	+	+	−	−
99	0·92	0·50	0·95	0·08	0·56	0·49	0	6	+	−	+	−	+	+	+	+	+	−	−
00	0·50	0·20	0·58	0	0·33	0·13	0	6	+	−	+	−	+	+	+	+	+	+	−

*, †, ‡. See Table 1. § Bright red pigment (water insoluble).

grew well when the NaCl level in artificial sea water was reduced to 0·2 M; six of the 15 showed no growth in NaCl levels above 0·8 M. Thus, these isolates were clearly distinct from the widely studied halophilic group whose growth has been shown to be inhibited at salt levels below about 2·0 M, yet constitute a 'halophilic' group growing optimally at salt levels near that in sea water.

Table 5. *Salt tolerance of selected marine bacteria*

Isolate no. M.B.	Relative growth* in 0·1 % trypticase broth with indicated NaCl concn. (M)					
	0·2	0·4	0·8	1·4	2·0	2·6
3	++	++	+	0	0	0
13	++	++	++	0	0	0
19	++	+++	++	++	++	0
22	++	+++	++	0	0	0
29	++	++	++	+++	+++	0
31	+	++	++	++	+	0
34	++	++	+++	++	+	0
43	+++	++	++	0	0	0
58	+	+	+	+	+	0
60	++	++	++	0	0	0
73	++	++	++	++	++	0
83	++	++	++	++	++	0
84	++	++	++	++	++	0
98	++	++	++	+++	+++	0
100	++	++	++	0	0	0

* Judged by visual turbidity in cultures incubated 48 hr. on a shaker at 30°. All media contained KCl, $MgCl_2$ and $MgSO_4$.

DISCUSSION

Two properties seem particularly to demonstrate a close interrelationship among the 96 isolates: the narrow range of salinities tolerated, and the universal requirement for Na^+ for growth. Failure of any isolate to multiply in the absence of added salt places the group in the category of obligate halophiles; yet they do not precisely fit the classifications either of Flannery (1956) or of Ingram (1957). In the former, they would be excluded by growing well in media with less than 0·3 M-salt, and in the latter, they would be included with a group (IIIA) characterized by growth in media containing salt up to well above half-saturation. All of the isolates required some NaCl in the medium to grow well; preliminary experiments have shown that some, but not all, of the NaCl requirement may be replaced by an osmotic substitute such as sucrose. This requirement for Na^+ at more than trace levels may be a distinctive property of these marine bacteria (see MacLeod & Onofrey, 1957).

Certain correlations of properties became apparent when the isolates were grouped on the basis of mineral requirements. Carbohydrate fermentation was observed with 19 of the 25 isolates which required only $Na^+ + K^+$ and with 17 of the 19 which grew well with Na^+ as the only added cation. In contrast, only eight of the 52 isolates showing a specific requirement for Mg^{++} fermented one or two of the substrates tested by the technique used. Similarly distributed was the capacity to produce nitrite from nitrate: 17 of the $Na^+ + K^+$, and eight of the Na^+ group possessed this ability, while only four of the remaining 52 isolates produced nitrite. Indole production was limited to members of the $Na^+ + K^+$, and Na^+ groups, 11 occurring in the former, and three in the latter. H_2S production, occurring with only 11 isolates, was also restricted largely to the $Na^+ + K^+$ and Na^+ groups, with only one isolate showing this property among the other groups. These observations suggest that physiological versatility is a more common feature of marine isolates whose mineral nutrition can be satisfied by adding only Na^+, or $Na^+ + K^+$ than of those requiring Mg^{++} as well. At the same time, it must be noted that some representatives of the 'versatile' group (e.g. M.B. 58, M.B. 95) were as limited physiologically, according to these criteria, as any members of the other group.

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Studies upon an Oral Aerobic Actinomycete

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SUMMARY

An aerobic actinomycete isolated regularly from the human mouth is described. This organism possesses the cultural, morphological and biochemical characters of Ørskov's Group 2b, or more specifically, the genus *Nocardia.* Although the results of cell-wall analyses differ from those obtained in this and previous studies upon other species of *Nocardia*, we nevertheless feel that provisional classification in the genus *Nocardia* is valid upon the present evidence. The specific name *N. salivae* is proposed.

INTRODUCTION

Gram-positive bacteria which exhibit morphological features of the order Actinomycetales occur commonly and regularly in the human mouth (Bisset & Davis, 1959). Morphological criteria are of prime importance in the subdivision of this order into families and genera, although many attempts have been made to supplement this approach with biochemical, serological and other information, with varying degrees of success. The main point which emerges from any close examination of this large group of bacteria is that here, as in many other groups, the lines of demarcation between genera are not clear. For instance, if one examines typical strains of *Streptomyces*, *Actinomyces*, *Nocardia*, and *Mycobacterium*, it appears obvious that *Streptomyces* is characterized by its permanent mycelium, aerial hyphae and chains of spores, *Actinomyces* by its anaerobic tendencies, *Nocardia* by its fragmenting impermanent mycelium, and *Mycobacterium* by its small size and acid-fastness, among other characters. In nature, however, there commonly occur strains of all these genera which not only fail to exhibit the more obvious characteristics of the genus to which they are assigned, but which appear to possess characteristics of other genera. Examples of this are: mycobacteria which produce mycelium and are not acid-fast; nocardias which form aerial mycelium and spores, or which simulate mycobacteria; actinomyces which grow aerobically, with rudimentary aerial hyphae; streptomyces without aerial hyphae or spores. The various morphological appearances which the Actinomycetales produce can in fact be almost as great a taxonomic hazard as they appear to be a blessing. For example, Jensen (1932) pointed out that the sporophores of *Micromonospora* closely resemble similar structures in 'Actinomyces' (i.e. *Streptomyces*) and 'Proactinomyces' (i.e. *Nocardia*); Bisset (1957) also compared these structures with similar ones in *Actinomyces odontolyticus*, a microaerophilic parasite, and discussed the probable significance of these similarities. In addition to this type of apparent cross-relationship between relatively well-established genera of the order Actinomycetales, the taxonomy of the group is further complicated by the occurrence of a number of organisms which possess certain of the group characteristics but which are not currently generally accepted as members of the group, such as *Lactobacillus bifidus*, *Propionibacterium*, *Corynebacterium*, *Arthrobacter*, *Leptotrichia*, etc. (see *Bergey's Manual*, 1957).

Of the genera which are accepted as members of the Actinomycetales, *Nocardia* is one of the largest and most poorly classified. Members of this genus occur most commonly in soils, but various pathological conditions in man and other animals have been associated with certain species. Considerable variation in morphological and biochemical potential is characteristic of the genus *Nocardia* as it is currently defined.

In 1954 Morris pointed out that many of the so-called *Actinomyces* of the human oral flora were more accurately classified as *Nocardia*; he described a number of types but was unable to identify them with any known species, most of which are relatively poorly defined. The dental literature abounds with references to 'actinomyces' and 'actinomyces-like bacteria' (Rosebury, 1944), particularly in relation to the problem of dental calculus formation, e.g. Naeslund (1926). Most of these reports are difficult to evaluate taxonomically because inadequate descriptions were made. An idea of the associations between actinomycetes and oral conditions can be gathered from the list given by Dodge (1936); some of the oral types (all called 'Actinomyces') described in that work are clearly very streptomyces-like, e.g. '*A. buccalis*', and others may be assumed, but not proved, to be nocardias, e.g. '*A. Rodellae*'.

In many references to oral and other organisms of the *Actinomyces-Nocardia* type the problem of generic allocation has been circumvented by resorting to the designation used by Ørskov (1923) as 'members of Group 2*b*'. Ørskov's Group 2*b* included those nocardias which do not simulate *Streptomyces* and also the microaerophilic types now called *Actinomyces*. This grouping reflects the way in which the two genera *Nocardia* and *Actinomyces* tend to merge together, especially in morphological characters. But this is not the only direction in which *Nocardia* appears to merge into another genus; it does so with both *Mycobacterium* and *Streptomyces*. It is interesting, and somewhat disconcerting, to note that *Actinomyces*, *Streptomyces* and *Mycobacterium* are not only currently classified as separate genera, but also in separate families, and yet with all three *Nocardia* shows certain affinities, or appears to do so. Various possible explanations for this situation are available, the most likely being either that *Nocardia*, in its various forms, represents facets of the primitive, basic actinomycete from which the other forms have evolved, or that *Nocardia* represents a collection of semi-degenerate streptomyces, micromonosporas, actinomyces, etc. Considering the apparent specialization exhibited by most other genera of the order Actinomycetales it seems that the former hypothesis is more appropriate. The present work does not set out to prove or disprove this hypothesis, but only to provide taxonomically useful information.

Some information on the organism described here has already been published (Davis & Baird-Parker, 1959*b*).

RESULTS

Isolation and Culture of Nocardia salivae

Isolates of the organisms to be described and which it is proposed to name *Nocardia salivae* were first obtained during the process of isolating *Leptotrichia dentium* (Baird-Parker & Davis, 1958; Davis & Baird-Parker, 1959). Submerged cultures of saliva and *materia-alba* were made in NAYS agar (composition: nutrient broth containing 0·3% (w/v) yeast extract, 0·2% (w/v) soluble starch and 1% (w/v) agar, pH 7·0). After incubation for 2–3 days at 37° colonies were selected (see section on Morphology) with the aid of a low-power dissecting microscope, and subcultured in further pour plates and on the surface of NAYS agar. With a little experience it was easy to distinguish between colonies of the nocardia-like organism here described and those of *Leptotrichia dentium*, the latter being like balls of hair in submerged culture and giving smooth, heaped-up colonies with irregular (lobate) and noticeably filamentous margins in surface culture. The grossly filamentous nature of *L. dentium* is in fact characteristic, whereas the nocardia-like organism has never shown this feature in its colonies. Subsequently most isolates were obtained from aerobic surface cultures upon NAYS agar or blood agar. Strains of *N. salivae* were obtained from almost all the mouths examined.

Growth of *Nocardia salivae* in nutrient broth sometimes failed or was very slow but was usually fairly good and produced a smooth turbidity with small floccules. In semi-solid nutrient agar (0·3%, w/v, agar) the nocardias produced smooth confluent growth on the surface. Growth upon nutrient agar was often adequate in 24 hr., and this faster growth rate served to distinguish further between strains of *N. salivae* and those of the slower growing *Leptotrichia dentium*. Strains of *N. salivae* did not grow under strictly anaerobic conditions but were satisfactory in air and air +10% (v/v) CO_2. Good growth was obtained upon inspissated serum, Lowenstein-Jensen medium, liver extract agar, Dorset's egg medium (with and without glycerol), 5% horse blood agar, potato-nutrient agar (Waksman, 1950), and at and near the surface of Brewer's medium. Poor growth occurred on oatmeal agar (Waksman, 1950), and no growth on glucose-asparagine agar, or in gelatin stab cultures. When growth occurred its character was basically similar on all media. Of the chemically defined media tested only that of Gray & Thornton (1928) supported some growth; addition of glycerol or glucose (1%) or a drop of liquid paraffin/tube (see Umbreit, 1939) improved growth.

It was noticed that *Nocardia salivae* grew more readily upon solid than liquid media and an effort was therefore made to evolve a satisfactory liquid medium. It was found that nitrate stimulated growth and by mixing the salt solution of Czapek-Dox with nutrient broth a very satisfactory medium was obtained. This mixture was later modified to the following (g./100 ml. water): Lab Lemco, 0·5; peptone (Oxoid), 0·5; $FeSO_4$,0·001; NaCl, 0·05; $MgSO_47H_2O$, 0·05; K_2HPO_4, 0·1; KNO_3, 0·1; adjusted to pH 7·0. This medium will be called Lemco salts medium (see section on Biochemical characters).

During growth of *Nocardia salivae* in Lemco salts medium the pH value rose to *c.* 8·0 in 24 hr., but when glucose or sucrose was present this initial pH increase was followed by a decrease to *c.* pH 5·2 (2% glucose) in 3 days. Whether the initial pH increase was due to ammonia production from nitrite or from some protein constituent was not ascertained. Traces of ammonia were detected in nitrite reduction tests using Nessler's reagent, but none was detected in tests with arginine hydrochloride as potential source of ammonia. Growth in broth cultures was often adherent to the tube walls and this character was significantly increased in broths containing fermentable carbon sources. It was also noted in test broths adjusted to pH 5·5 with hydrochloric acid in the absence of carbohydrate, and is probably related to growth at low pH values. Recent tests with a solid version of Lemco salts medium indicate that it may prove useful as a semi-selective primary isolation medium for oral nocardias; this aspect will be studied further.

Morphology of Nocardia salivae

Colonial form. Submerged colonies in NAYS agar are small, discrete, very dense and irregular in outline, resembling a grain of sand or soil crumb.

Surface colonies (see Pl. 2) on NAYS agar vary considerably in appearance. They commonly commence as small, granular, entire, slightly raised colonies which often develop a dark central spot (seen when viewed by transmitted light). Older growth is relatively flat, dry and wrinkled, often curling away from the substrate at the margins to produce a lily-leaf like appearance (Pl. 2, fig. 14). In some cases growth is relatively smooth, soft and entire (Pl. 2, fig. 7), and in others extremely rough, irregular, hard and sunk into the substrate (Pl. 2, fig. 15). Intermediate granular, segmenting colonies commonly occur (Pl. 2, figs. 10, 11 and 13). These various colonial forms appear to be related to the type of cell forms in them, the smooth and granular forms being largely composed of coccoid and short forms and the heaped-up, adherent rough types of more filamentous cells.

On primary isolation plates colonies may appear to be erupting from the agar as rosettes (see Pl. 2, fig. 15), or lying almost free upon the surface as rough granules.

Adherence to the substrate is common, and usually results in colonies with crimped edges, somewhat resembling the primary growth of *Streptomyces*. These colonies are, however, seldom as large or as solid in consistency as those of *Streptomyces*, being usually composed of a mealy or crumbly top and adherent base. In many cases complete colonies can be removed from the agar without breaking up; in others the colonies fragment in a brittle fashion when disturbed. Colonies are invariably creamy white in colour; no sign of aerial mycelium formation was detected. We should like to draw attention to the similarities between the colonial forms of *Nocardia salivae* and those recently described by Howell, Murphy, Paul & Stephan (1959) in oral *Actinomyces* spp.

Cellular form. Nocardia salivae exhibits a wide range of cell forms and arrangements of the type normally associated with members of the genus *Nocardia.* It is always Gram-positive and motility has not been observed. The cells are robust and vary considerably in diameter.

In Fig. 1 the range of cell forms is indicated by drawings; the sequence from A to W is not intended as a cycle of development; the arrangement merely shows the simpler forms first and more complex last. In some cases, however, it does seem likely that adjacent forms represent parts of a development cycle, e.g. A produces B; S produces T which fragments to form U, etc. One of the striking features of the simpler cell forms illustrated in Fig. 1, notably A, B, D, E, F, G, K, V, is their similarity to the cell forms described for *Arthrobacter* spp. (Conn & Dimmick, 1947).

The 'cystites' and 'tripodal cystites' of Chaplin (1957) are clearly simulated in drawings A, G, K and V, as also are the 'opposite' and 'adjacent angles' of Sundmann (1958) in drawings F and D (see also Pl. 1, figs. 2, 4). It is interesting to note here that in the newly introduced comprehensive key in *Bergey's Manual* (1957), Skerman points out (p. 1009) the very close similarity between *Arthrobacter* and *Nocardia.* Attention is also drawn to the similarity between the reported 'gonidia' formation in *Arthrobacter* (Chaplin, 1957) and the frequent reports of apparently similar processes in *Nocardia* (*Bergey's Manual*, 1957, p. 715), *Leptotrichia* (Bulleid, 1924), and *Streptomyces* (Dreschler, 1919); such forms were not common in the present organism, but in Pl. 2, fig. 9, an appearance which is grossly similar is shown, i.e. apparent coalescing of the cytoplasm within the cell wall. Similarities between certain cell forms in Fig. 1 and those reported by

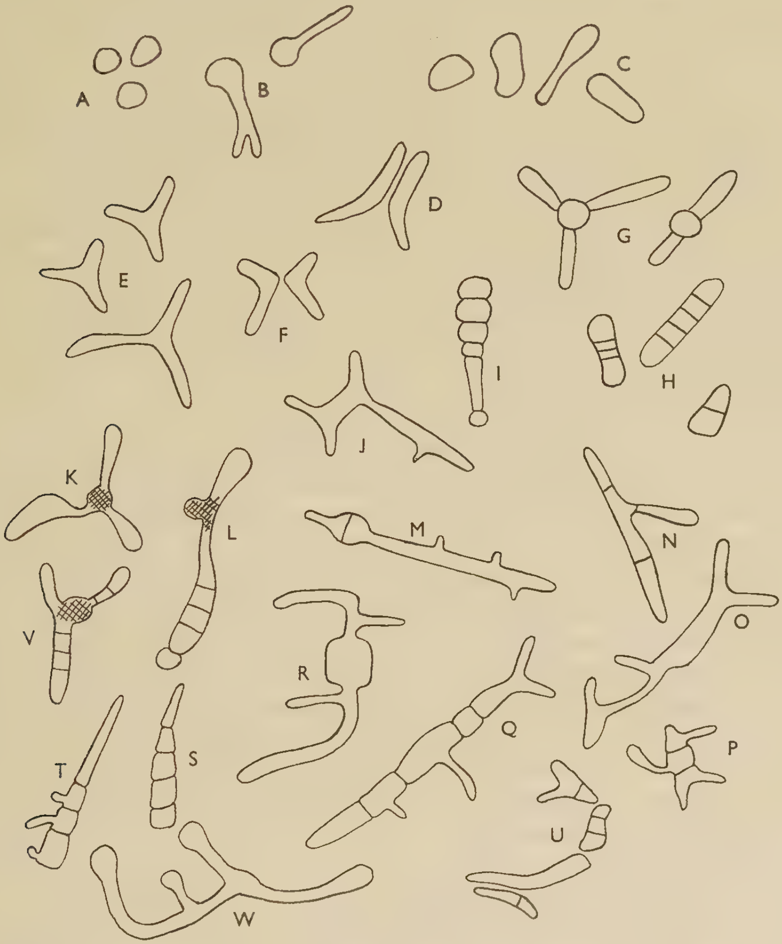

Fig. 1. Drawings of representative cell forms exhibited by the oral aerobic actinomycetes (*Nocardia salivae*) of this study, observed in preparations stained to show cell walls. Cross-hatching (K, L, V) indicates occurrence of densely staining areas.

Morris (1951) as stages in the life cycle of *Actinomyces bovis* (see Morris's fig. 1, stages 1–7, 11–23) are noticeable, as also are those between the figs. 5 and 20, 21 of Webb, Clark & Chance (1954) and Fig. 1 D and H, respectively, of the present work.

Filament formation is common (Pls. 1 and 2, figs. 1, 5, 8, 9 and 12), as is branching (Pl. 1, figs. 1, 5) and segmentation followed by the production of buds (Pl. 1, fig. 3); although budding preceding septation (Pl. 1, fig. 6) and leading to branching (Pl. 1, fig. 1) also occurs. Terminal swelling of filaments (Pl. 2, figs. 8, 9 and 12) and swollen short forms (Pl. 1, fig. 4) are common. The triradiate (see 'tripodal cystites' above) and short-paired angle forms (see also above) characterize this organism when growing in the short mycelial form. Although long branching filamentous forms are produced, mycelium formation is only rudimentary and not comparable with that which occurs in *Streptomyces* or *Nocardia asteroides* (see Bisset, 1955, p. 96). Preliminary attempts to follow the morphological development cycle of these bacteria were unsuccessful. The cells appear capable of reproducing indefinitely in certain forms, e.g. coccoids, over periods of days. It is, however, clear that many of the cell forms interpreted by previous workers as stages of life-cycles in *Nocardia* and *Actinomyces* can be observed in this bacterium. It is our opinion that the morphological potential of members of the order Actinomycetales constitutes a wide and, to a large extent, overlapping series of cell forms, and that the present organism exhibits many of those which place it taxonomically in the centre of the *Nocardia* range.

Biochemical characters of Nocardia salivae

Two series of tests were conducted, with an interval of six months between. In the first series 10 strains were used and in the second 20, five of which were strains from the first series. Some of the strains used were relatively fresh isolates while others had been in cultivation for one and a half to two years; no differences were noticed between fresh and old isolates, or in the results they gave. Owing to the poor growth obtained in liquid cultures in the earlier stages of this study (see section on Culture), the first series of tests were made on solid media whenever possible, e.g. fermentation tests in stab cultures, though nitrate and nitrite reduction, hippurate and aesculin hydrolysis and glucose utilization, etc., were tested in liquid media. The basal medium (pH 7·0) consisted of nutrient broth +0·3 % (w/v) yeast extract. In the second series of tests Lemco salts broth (see section on Culture) was used as the basal medium, and most tests, including fermentations, were made in liquid medium.

The two series reported were not exact duplicates in respect of tests carried out, technique of testing or results, and discrepancies are noted in the relevant tests by referring to the first series as *A* and the second as *B*. The fractional figures indicate strains positive/strains tested.

Catalase. On NAYS agar (*A* and *B*) positive: on Lemco salts agar (*B*) positive.

Nitrate reduction. 0·05 % (w/v) potassium nitrate and 0·1 % (w/v) agar added to broth, 7 days: *A* 9/10 positive; *B* positive.

Nitrite reduction. 0·01 % (w/v) potassium nitrite and 0·1 % (w/v) agar added to broth, 7 days: *A* 9/10 positive; *B* (with Lemco salt base without KNO_3) 18/20 positive.

Hydrogen sulphide production. A 0·05 % (w/v) L-cysteine hydrochloride in agar slopes, lead acetate paper, 7 days: *A* and *B* negative.

Hippurate hydrolysis. 1 % (w/v) sodium hippurate in liquid medium, tested with 50 % sulphuric acid, 7 days: *A* 2/10 positive, *B* negative.

Aesculin hydrolysis. 0·5 (w/v) aesculin and 0·05 % (w/v) ferric ammonium citrate: *A* and *B* positive 1–3 days.

Arbutin hydrolysis. B only, tested as with aesculin: positive but slower than aesculin, *c.* 5 days.

Glycerol utilization. A and *B* using stab cultures + pH indicator (Bromocresol purple) and 1 % (v/v) glycerol: acid production readily apparent at agar surface only; when sealed with liquid paraffin (*B* only) no acid production. Tests in liquid medium (*B*) gave very slow positive results, i.e. 15–25 days, but heavy growth. (See section on Culture).

Final pH *value in* 2 % (*w/v*) *glucose broth. A* and *B*, pH tested over 7-day period, from second day onward pH was 5·2; *A* only with 1 % (w/v) glucose pH 5·8–6·0.

Acetylmethylcarbinol production. A, with 1 % (w/v) glucose, 1/10 positive: *B*, with 2 % (w/v) glucose, 19/20 positive. Tested by Barritt's method (note probable effect of increased glucose concentration in *B*).

Litmus milk. B only, no reaction in 10 days.

Gelatin liquefaction. Method of Smith (1946): *A* and *B*, positive.

Starch hydrolysis. Starch 0·2 % (w/v) in agar plate method: no or extremely slight hydrolysis.

Growth in chemically defined liquid media. Czapek-Dox negative or very slight; Dulaney (1948) medium B, negative; Gray & Thornton (1928), usually weakly positive. See section on Culture for other media.

Carbohydrate fermentations. Concentrations 1 % (w/v), observed over 21 days: glucose, maltose, fructose, sucrose, trehalose, glycerol and arbutin (*A* only) fermented by all strains in *A* and *B*. Mannose fermented by 2/10 strains in *A*, by all strains in *B*. Galactose, lactose, arabinose, xylose, rhamnose, melibiose, melezitose, raffinose, dextrin, inulin, glycogen, starch, dulcitol, erythritose, mannitol, sorbitol, inositol, cellobiose, salicin not fermented. No gas was produced from any sugar (*B* only). Indicator changes in series *A*, i.e. stab cultures, occurred only at and near the surface of the medium and suggest oxidative rather than fermentative dissimilation of carbohydrates.

Acid-fastness. Organisms from NAYS agar were resistant to 1 % sulphuric acid, rarely resistant to 5 % and never to 20 %.

The following tests gave totally negative results: casein digestion, with skimmed milk agar; tyrosine and xanthine digestion; tolerance of 10 % sodium taurocholate; haemolysis of horse blood; indole production; citrate utilization. No pigment production or discoloration of the agar was observed on any medium.

Cell-wall analyses

Cell-wall hydrolysates for amino acid analyses were prepared as previously reported (Davis & Baird-Parker, 1959*b*). Cell breakage with ballotini using a 'Microid' wrist-action shaker was also tested and yielded results comparable to those from organisms broken by the ultrasonic method.

Chromatography. Two-dimensional descending paper partition chromatography was used to analyse the amino acid constituents. The organic phase of the mixture—*n*-butanol (4), glacial acetic acid (1), water (5)—was used as first solvent (16 hr.);

and pyridine (50), ethanol (35), water (15), as the second (6 hr.). Runs were made upon Whatman no. 1 paper in lagged tanks. Papers were air dried and sprayed with 0·25 % (w/v) ninhydrin solution (in 95 % acetone). The addition of a trace of collidine to this reagent was found helpful in that it causes certain amino acids to develop as distinctively coloured spots after heating 5 min. at 105°.

The method of Hoare & Work (1957) was used to distinguish the isomers of diaminopimelic acid.

Table 1. *Cell-wall amino acid patterns determined in this study*

Strain	LL-DAP	DL- or DD-DAP	Lysine	Aspartic acid	Glutamic acid	Alanine	Glycine	Serine	Valine	Leucine	'Under Aspartic'	Hexosamines
Nocardia salivae												
N 5	–	–	++	Tr.	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
N 2	–	–	++	+	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
N 1	–	–	++	+	++	++	Tr.	Tr.	Tr.	+	–	Tr.
P 1	–	–	++	+	++	++	+	+	+	+	–	Tr.
G 5A	–	Tr.	++	+	++	++	+	+	+	+	–	Tr.
T 3	–	–	++	Tr.	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
T 1	–	+	++	+	++	++	+	Tr.	+	+	–	Tr.
X 1	–	–	++	+	++	++	+	–	Tr.	+	–	Tr.
M 1	–	–	++	+	++	++	+	+	Tr.	+	–	Tr.
JL 1	–	–	++	+	++	++	Tr.	–	Tr.	+	–	Tr.
B 4	–	–	++	Tr.	++	++	+	+	Tr.	+	–	Tr.
DE 1	–	–	++	Tr.	++	++	–	–	Tr.	+	–	Tr.
Leptotrichia dentium												
LD	–	++	–	Tr.	++	++	Tr.	–	Tr.	Tr.	–	Tr.
V 1	–	++	Tr.	+	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
L. buccalis												
1	–	++	Tr.	Tr.	++	++	Tr.	Tr.	Tr.	+	Tr.	Tr.
2	–	++	Tr.	+	++	++	Tr.	Tr.	Tr.	+	–	–
3	–	++	+	+	++	++	+	Tr.	Tr.	+	Tr.	–
Lactobacillus plantarum												
3 D	–	++	Tr.	+	++	++	+	Tr.	Tr.	Tr.	–	–
3 C	–	++	Tr.	Tr.	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
L. casei												
2 I	–	–	++	+	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
2 G	–	–	++	+	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
2 A	–	++	Tr.	+	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
Streptomyces sp.												
S 25	++	–	–	+	++	++	+	Tr.	Tr.	Tr.	–	Tr.
Streptomyces sp.												
S 26	++	–	Tr.	+	++	++	+	Tr.	+	+	–	Tr.
Propionibacterium pentosaceum NCIB 8070	++	–	Tr.	+	++	++	+	Tr.	Tr.	Tr.	–	Tr.
Nocardia asteroides												
NCTC 8595	–	++	+	+	++	++	+	Tr.	Tr.	+	–	–
Corynebacterium fascians	–	++	Tr.	Tr.	++	++	Tr.	Tr.	Tr.	Tr.	–	Tr.
C. hofmanii												
NCTC 231	–	++	Tr.	+	++	++	+	+	+	+	–	Tr.
Soil nocardia												
C 1	–	++	Tr.	Tr.	++	++	Tr.	–	Tr.	Tr.	–	–
Jensenia sp.	–	++	Tr.	+	++	++	+	+	+	+	–	Tr.

++ = major component; + = minor component; Tr. = trace; – = not detected.

In addition to 12 strains of *Nocardia salivae*, 2 of *Leptotrichia dentium*, 3 of *L. buccalis*, 1 of *Corynebacterium fascians*, 1 of *C. hofmannii* (NCTC 231), 1 of *Jensenia* spp., 1 of *Propionibacterium pentosaceum* (NCIB 8070), 1 of *Nocardia asteroides* (NCTC 8595), 1 soil nocardia and 2 soil streptomyces (C 1, S 25, S 26 respectively, of Davis & Baird-Parker, 1959 *b*), and strains of *Lactobacillus casei* and *L. plantarum*, were examined for comparative purposes.

The results are shown in Table 1; in Table 2 the results for the oral aerobic actinomycetes are compared with certain relevant results from Cummins & Harris (1956, 1958, 1959*a*, *b*), and are discussed later.

Three points arose from the cell-wall studies which do not bear directly upon the present problem but which require mention. First, the finding of an unidentified component ('under aspartic') in two of the three extracts of *Leptotrichia buccalis*, but not in those of *L. dentium*, suggests a possible taxonomic distinction. Secondly, we have examined nine strains of *Lactobacillus casei* which were available in this laboratory and found three of them to possess DAP rather than lysine in the cell wall. In a previous publication (Davis & Baird-Parker, 1959*b*) the predominance of DAP in a single strain of *L. casei* was reported and when doubt was thrown upon the accuracy of this result it was checked, unfortunately, by the use of the other two strains which possess mainly DAP. We have not examined these strains of *L. casei* to determine the cause of these apparently aberrant results but now report that in our experience two out of three strains of *L. casei* yield results comparable with those reported by Cummins & Harris (1956), and dissimilar to the results obtained from *L. plantarum* (see Table 1). Thirdly, the organisms named *Jensenia* (Bisset & Moore, 1949) and *Corynebacterium fascians* (see Lacey, 1955) both exhibit cell-wall amino acid patterns of the *Mycobacterium–Corynebacterium–Nocardia* type.

DISCUSSION

The most significant feature of the results described above is the apparent taxonomic contradiction between the results of morphological, cultural and biochemical studies and those of the cell-wall analyses of *Nocardia salivae*. Upon the evidence of the former these oral bacteria are classifiable in the genus *Nocardia*. They possess the characteristic colonial and cellular features of the genus, are aerobic, catalase-positive and exhibit biochemical and cultural characters of the *Nocardia* type. Comparison with descriptions given in *Bergey's Manual* (1957), *Topley and Wilson's Principles* (1955), Waksman (1950), and similar texts, clearly indicates the genus *Nocardia*. The results of the cell-wall analyses do not, however, support this classification (see Davis & Baird-Parker, 1959*b*) and appear to more closely resemble those obtained from the microaerophilic *Actinomyces* (see also Cummins & Harris, 1959*b*).

Table 2 compares the cell-wall analysis results of Cummins & Harris (1956, 1958, 1959*a*, *b*) with those obtained in this laboratory with various organisms; from this and Table 1 the overall similarity between the results, particularly as regards major components, is readily seen. The quantitative evaluation of hydrolysate components upon essentially qualitative chromatograms is necessarily arbitrary and for this reason it is not possible to consider the occurrence of aspartic acid, glycine, serine, valine, leucine, etc., in our results and their absence, or omission, from the results of Cummins & Harris, as highly significant. Consequently it seems more

Table 2. *Comparative cell-wall analysis results for various genera, etc., based upon Cummins & Harris* (1956, 1958, 1959) *and work in this laboratory*

	LL-DAP	DL-or DD-DAP	Lysine	Glutamic acid	Alanine	Aspartic acid	Glycine	Serine	Valine	Leucine	Glucosamine	Galactos-amine	Muramic acid	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Fucose
*Micromonospora**	++	Tr.	–	++	++	–	+	NR.	NR.	NR.	Tr.	–	Tr.	–	–	–	–	–	–
Streptomyces	++	–	–	++	++	+	+	Tr.	Tr.	Tr.	Tr.	–	Tr.	–	–	–	V	–	–
Propionibacterium	++	–	–	++	++	+	+	Tr.	Tr.	Tr.	Tr.	V	Tr.	–	V	V	V	V	–
Leptotrichia	–	++	–	++	++	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	–	NR.	–	–	–	+	–	–
Nocardia	–	++	–	++	++	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	–	Tr.	+	–	+	V	V	–
*Mycobacterium**	–	++	–	++	++	–	–	NR.	NR.	NR.	Tr.	–	Tr.	+	–	+	V	V	–
Corynebacterium I	–	++	–	++	++	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	V	Tr.	+	–	+	V	V	NR.
Corynebacterium II*	–	–	++	++	++	–	–	–	NR.	NR.	Tr.	Tr.	Tr.	–	+	–	+(V)	V	NR.
*Streptococcus**	–	–	++	++	++	–	–	–	NR.	NR.	Tr.	Tr.	Tr.	–	+	V	V	V	NR.
*Actinomyces bovis**	–	–	++	++	++	+	–	NR.	NR.	NR.	Tr.	–	Tr.	–	+	V	–	–	V
NCTC 4500 and 4502*	–	–	++	++	++	+	+	+	NR.	+	Tr.	Tr.	Tr.	NR.	NR.	NR.	NR.	NR.	NR.
*Actinomyces israelii**	–	–	++	++	++	–	–	NR.	NR.	NR.	Tr.	–	Tr.	–	–	+	–	–	–
Nocardia salivae	–	–	++	++	++	Tr.	Tr.	Tr.	Tr.	Tr.	...	Tr.	...	–	–	+	–	–	NR.
Lactobacillus casei	–	–	++	++	++	+	Tr.	Tr.	Tr.	Tr.	...	Tr.	...	Tr.	+	+	+	+	NR.
Arthrobacter spp.*	–	–	++	++	++	NR.	–	NR.	NR.	NR.	Tr.	V	Tr.	—	NR.	+	–	Tr.	NR.

++ = major component; + = minor component; Tr. = trace; NR. = not reported; * = results of Cummins & Harris only; V = variable; – = not detected.

Note. Cummins & Harris (1956) concluded that strains here called *Corynebacterium* II may be streptococci.

reasonable to compare our results for oral nocardia-like bacteria with the *Actinomyces israelii* results of Cummins & Harris rather than those for *A. bovis* or the NCTC strains 4500 and 4502 in which Cummins & Harris reported aspartic acid, glycine and serine, among others, as additional constituents. Apart from this point, the presence of galactose (see Davis & Baird-Parker, 1959 *b*) but nor hamnose or fucose in the present oral strains also suggests closer similarity to *A. israelii.*

Bearing in mind the comments made above about the morphological similarity between the organisms here described and *Arthrobacter* spp., it is of great interest that Cummins & Harris (1959*b*) reported cell-wall analysis results for five out of seven named species of *Arthrobacter* as identical with those from *Actinomyces israelii.* This tempts us to suggest that *Arthrobacter, Nocardia salivae,* and *Actinomyces* may represent a related series of bacteria ranging from saprophytes to parasites. The genus *Arthrobacter* is already somewhat of a heterogeneous group of species held together by certain common morphological characters. Unfortunately the heterogeneity in cell-wall composition noted by Cummins & Harris (1959*b*) does not correlate with that noticeable in other characters (*Bergey's Manual,* 1957). In view of certain dissimilarities, notably biochemical, between the organisms we have studied and the known characters of *Arthrobacter* species as defined at present, there seems little point in increasing the heterogeneity of *Arthrobacter* by classifying our organisms in this genus. We consider it unwise at this stage to transfer apparently anomalous species from one genus, e.g. *Nocardia,* to another, e.g. *Arthrobacter,* in which they may prove to be equally out of place when better understanding of this group of organisms is obtained. Upon the basis of amino acid patterns comparison is also possible with the results for *Lactobacillus casei* (Cummins & Harris, 1958, noted the similarity between amino acid patterns from lactobacilli and *Actinomyces bovis*) and grossly similar patterns were reported by Cummins & Harris (1956) in *Streptococcus, Corynebacterium pyogenes* and *C. haemolyticum,* which also contain rhamnose as a major cell-wall component (*Corynebacterium* II of Table 2). Although these comparisons appear to confound rather than facilitate diagnostic classification the possible phylogenetic implications are interesting.

It is well known that the anaerobic character of *Actinomyces* is variable. In many classifications (Ørskov, 1923; Erikson, 1935) aerobic and anaerobic strains, i.e. *Nocardia* and *Actinomyces,* have been grouped together. The recent work of Howell *et al.* (1959) indicated a series of types within the genus *Actinomyces,* ranging from the relatively aerobic to the anaerobic (Table 3). In the absence of sharp cultural differences between aerobic and anaerobic actinomycetes of the *Nocardia–Actinomyces* range, it is now common, and probably sound practice to consider catalase-positive strains as *Nocardia* and negative ones as *Actinomyces.* When the organisms of this study were first isolated we tentatively labelled them as *Actinomyces naeslundii* (Thompson & Lovestedt, 1951) but now that this species has been more thoroughly described (Howell *et al.* 1959) various discrepancies are apparent and, in the absence of any satisfactory alternative, we allocate this oral actinomycete to the genus *Nocardia.* It is almost certain that this bacterium has been described before, but exact identification with any previous description has not been possible. Morris (1954) divided his oral nocardias (some of which were catalase-negative, according to his results) into eight types on the basis of fermentation reactions. Morris's type 7 appears fairly similar to the strains used in this study. The type 3*b* of Onisi & Nuckolls (1958) also resembled the present organisms. As in Morris's work, division into types was arbitrary and Onisi & Nuckolls divided their type 3*b* from 3*a* on the basis of lactose fermentation even though this division placed some catalase-positive strains in type 3*a* which these workers equated approximately with the genus *Actinomyces* (see Table 3).

The question of the taxonomic significance of catalase production in this group is an important one, and the general lack of information upon this subject renders comparison with many earlier reports of little value. It is, for example, very difficult to decide whether Thompson & Lovestedt (1951), Naeslund (1925), Lord & Trevett (1936), studied the organisms here described or the *Actinomyces naeslundii* of Howell *et al.* (1959).

Table 3. *Comparison between* Actinomyces israelii, A. naeslundii, *and oral aerobic actinomycete* (Nocardia salivae) *upon basis of the characters reported by Howell* et al. (1959)

	Actinomyces israelii 158 strains—5 types						
Characters used by Howell *et al.* (1959)	1 41 strains	2 66 strains	3 32 strains	4 11 strains	5 8 strains	*Actinomyces naeslundii* 42 strains	*Nocardia salivae* 25 strains
Catalase production	−	−	−	−	−	−	+
Indole production	−	−	−	−	−	−	−
Acetoin production	−	−	−	−	−	−	+
Nitrate reduction	50	50	42	36	43	88	+
10 % Bile tolerance	−	−	−	−	−	60	−
Fermentations (% strains positive)							
Glucose	100	100	100	80	90	90	100
Maltose	100	100	90	70	70	80	100
Sucrose	100	90	80	80	50	80	100
Raffinose	100	0	30	40	10	80	0
Lactose	100	100	90	0	0	60	0
Inositol	75	40	50	50	10	50	0
Salicin	10	0	20	40	10	10	0
Ribose	75	V	V	V	V	10	NT.
Inulin	0	50	40	0	20	10	0
Mannitol	75	80	0	0	70	0	0
Xylose	75	90	60	60	50	0	0
Arabinose	10	0	0	0	0	0	0
Glycerol	0	0	0	0	0	0	100
Melezitose	0	0	0	0	0	0	0
Growth in air	−	−	−	−	−	±	+
Growth in air $+CO_2$	±	−	−	−	−	+	+
Growth in N_2+CO_2	+	+	+	+	+	+	NT.

All figures in table represent percentage of positive strains. NT. = not tested; + = positive; − = negative; ± = weak; V = variable.

Note. Howell *et al.* acknowledge morphology as the primary diagnostic character in subdivision of *Actinomyces*.

The 'aerobic actinomyces' of Bibby & Knighton (1941) were very similar to *Nocardia salivae* and these workers pointed out that the study of the aerobic oral actinomycetes had been, and probably still is, considerably hampered by the general tendency to use only anaerobic culture techniques for organisms which appear to resemble *Actinomyces*.

Among other reports of aerobic actinomycetes isolated from man it is important to distinguish those which refer to *Streptomyces* or streptomyces-like nocardias, from those which refer to organisms of the type described in this paper. Erikson (1935) drew attention to the significance of 'powdery' colonies and its frequent lack of recognition by the workers who described them. The reports of Goadby (1903; 'Streptothrix buccalis'), Wright (1937), Franklin (1940) and Goldsworthy (1937) were of these streptomyces-like type of organisms. The *Nocardia buccalis* of Roger, Bory & Sartory (Castellani & Chalmers, 1919, p. 1061) also appears to be streptomyces-like but we have been unable to confirm this from the original description. Whether the so-called *Actinomyces buccalis* (NCTC 4523) represents this organism we do not know, but we have already reported on the streptomyces-like cell-wall nature of this organism (Davis & Baird-Parker, 1959*b*).

A feature of *Nocardia salivae* strains is their saccharolytic power. This character sets them apart from the typical soil nocardias, and appears to be typical of *Nocardia* (Von Magnus, 1947) and *Corynebacterium* strains (Conn & Dimmick, 1947; Laughton, 1950) which inhabit the mucous membranes of animals. It indicates that these strains represent ecological variants adapted to growth in these sites. Von Magnus (1947) suggested the name 'Lactomyces' for the acid-producing members of Ørskov's Group 2b, in order to indicate their intermediate taxonomic position between *Lactobacillus* and *Actinomyces*. We have already discussed this point, but upon the present evidence cannot come to any definite conclusion.

The general metabolic similarity between oral strains of *Nocardia*, *Actinomyces* (Pine & Howell, 1956), and *Leptotrichia* (Davis & Baird-Parker, 1959*a*) suggests that strong ecological pressures operate in the human mouth and that these members of the order Actinomycetales compete with, by simulating, the oral representatives of the Lactobacteriaceae, i.e. *Lactobacillus* and *Streptococcus*, for the available carbon sources. Preliminary attempts to determine the acid end-products of glucose metabolism by *Nocardia salivae* failed to detect lactic acid chromatographically, but this aspect will be pursued further.

Type strains of *Nocardia salivae* and *Leptotrichia dentium* have been sent to the National Collection of Type Cultures, Colindale Avenue, London, N.W.9, and allotted the numbers NCTC 10,207 (*Nocardia salivae*) and NCTC 10,206 (*Leptotrichia dentium*).

The authors wish to thank Dr A. C. Baird-Parker for his help in the early stages of this study.

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EXPLANATION OF PLATES

PLATE 1

Figs. 1–6. Oral aerobic actinomycete (*Nocardia salivae*) stained by the phosphomolybdic acid and methyl green method for cell walls.

Fig. 1. Branching filamentous forms. × 2,500.

Fig. 2. Triradiate and paired angle forms. × 1,250.

Fig. 3. Segmenting and budding filaments. × 2,500.

Fig. 4. Short swollen forms. × 1,250.

Fig. 5. Filamentous form with terminal bifurcation. × 2,500.

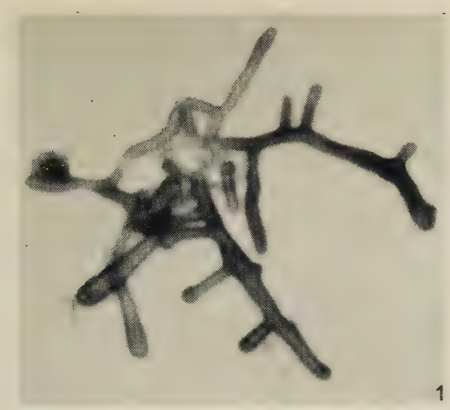
Fig. 6. Filament showing incipient budding. × 2,500.

PLATE 2

Figs. 7, 10, 11, 13, 14, 15. Colonies of *Nocardia salivae* on the surface of NAYS agar, showing various characteristic types. × *c.* 40.

Figs. 8, 9, 12. Filamentous cell forms in *N. salivae*, stained phosphomolybdic acid and methyl green for cell walls. The figures illustrate lateral branching (fig. 9), bifurcation (fig. 8), incipient bifurcation (figs. 9, 12), terminal (fig. 9), subterminal (fig. 12) and generalized (fig. 8) swelling of the filaments, and certain indeterminate cell inclusions (notably fig. 9). × 1,250.

Journal of General Microbiology, Vol. 23, *No.* 1 Plate 1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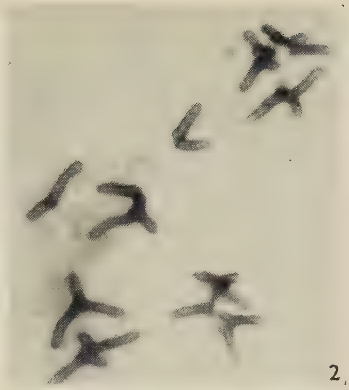
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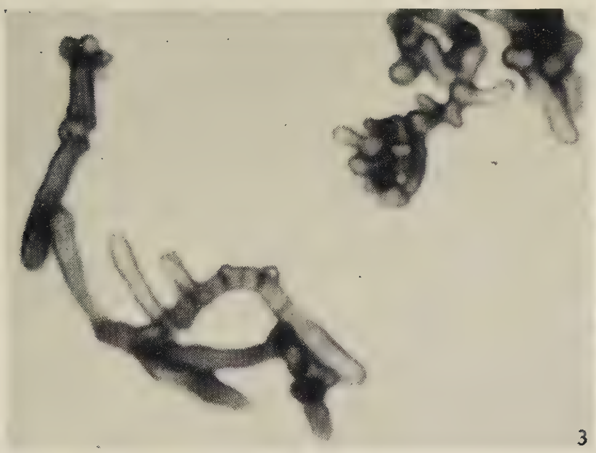
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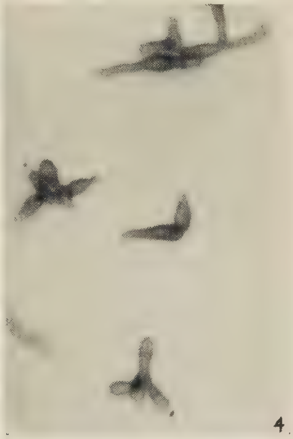
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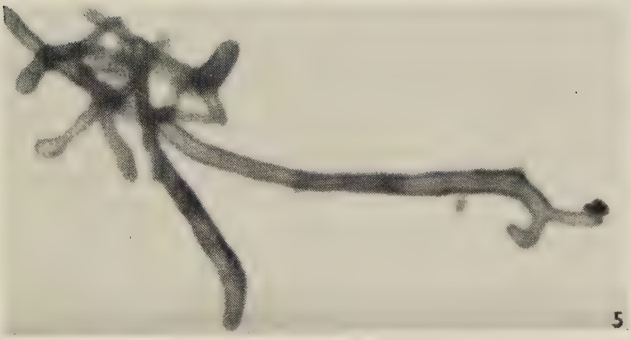
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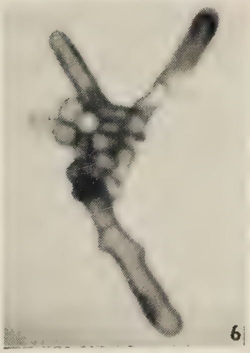
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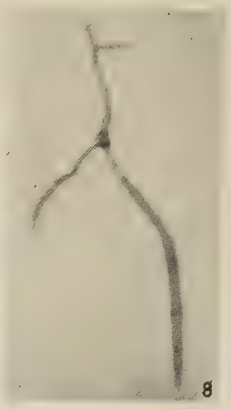
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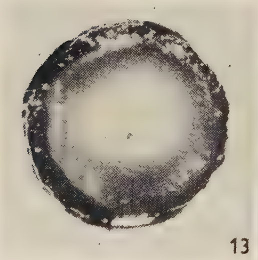
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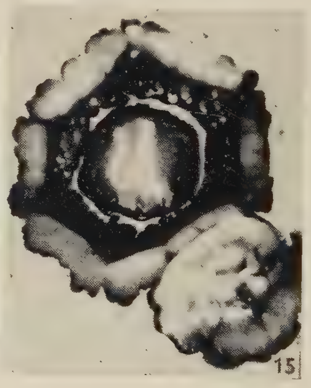
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The Bactericidal Action of Streptomycin on *Staphylococcus aureus* and Some Accompanying Biochemical Changes

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SUMMARY

Some factors which affect the killing by streptomycin of *Staphylococcus aureus* in growing cultures have been investigated. In short-term experiments, streptomycin is not bactericidal under conditions in which growth of the organisms cannot occur (low temperature, absence of nitrogen source, presence of growth-inhibitory concentrations of chloramphenicol). The rate of killing of organisms growing anaerobically is considerably lower than that of organisms growing aerobically. Streptidine can partially reverse the growth inhibitory effect of streptomycin. During the period in which growth becomes inhibited by streptomycin, the rates of incorporation of ^{14}C-labelled precursors into the major cell constituents, and of synthesis of nucleic acids, are reduced simultaneously, and no large changes occur in the total quantities of amino acids or 260 mμ-absorbing substances in the metabolic pool. During this period the rate of metabolism of glucose via the Embden–Meyerhof pathway is decreased, but there is no change in the proportion of the total glucose oxidized which is metabolized via a C_1-preferential oxidation. The Q_{O_2} value for oxidation of glucose by organisms harvested when inhibition of growth by streptomycin is first detectable is lower, by about 20 %, than that of normal organisms, and falls to *c.* 30 % of normal when growth is fully inhibited. At this time the Q_{O_2} values for oxidation of glycerol, lactate and formate are also reduced, by 40–80 %. These effects appear to be specific to organisms whose growth is inhibited by streptomycin.

INTRODUCTION

Many investigations have shown that streptomycin can affect numerous metabolic reactions in streptomycin-sensitive bacteria, both in growing and in non-growing organisms. It is not practicable to review all these observations here; early results have been discussed by Henry & Hobby (1949), and a very valuable discussion of more recent work has been given by Lightbown (1957).

A number of workers (Barkulis, 1953; Rosanoff & Sevag, 1953; Umbreit, 1953) have shown that, in non-growing organisms, streptomycin can inhibit certain reactions concerned in the metabolism of pyruvate in *Escherichia coli*, although the relationships between these inhibitory effects and the inhibition of growth has not been investigated. Hypotheses based on these and other observations have been discussed in detail by Lightbown (1957), particularly in relation to the antagonism between streptomycin and certain substituted quinoline *N*-oxides which can inhibit a reaction in the cytochrome system (Lightbown & Jackson, 1956). The increased resistance to streptomycin which is shown by facultatively anaerobic organisms when growing under low oxygen tensions (Bondi, Dietz & Spaulding, 1946; May, Voureka & Fleming, 1947; Lightbown, 1954, 1957) or in the presence of certain inhibitors of the cytochrome system (Lightbown, 1957) suggests that the growth-inhibitory action of streptomycin may depend on the operation of the cytochrome system. Lightbown (1957) has suggested that these phenomena may be due to the interrelationships, such as the Pasteur effect, between aerobic respiratory processes and other reactions of intermediate metabolism. An alternative hypothesis which would be compatible with these observations is that the access of streptomycin to its site of action is dependent on the presence of oxygen or on some aerobic metabolic process.

No published studies describing inhibitory effects of streptomycin on metabolic reactions have established that inhibition of the affected reactions is the primary cause of loss of viability of the organisms. Many investigations have been concerned with effects of streptomycin on suspensions of non-growing organisms. However, it has been shown (Wasserman, Lessner & West, 1954; Lightbown, 1957) that organisms from exponentially growing cultures are much more rapidly killed by streptomycin than are non-growing organisms. It is possible therefore that the processes which lead to loss of viability in the presence of streptomycin do not occur in non-growing organisms, and it may be more profitable to search for effects of streptomycin using growing organisms under conditions of maximum sensitivity to streptomycin. It is also possible that the properties of metabolic systems in non-growing organisms may differ both qualitatively and quantitatively from those ingrowing organisms; such a difference in the properties of the acetate-oxidizing system of *Escherichia coli*, and in its sensitivity to inhibition by streptomycin, has been shown by Vignais & Vignais (1954)

This paper describes experiments designed to determine the conditions under which *Staphylococcus aureus* shows greatest sensitivity to streptomycin, as a basis for investigations of biochemical changes during the inhibition of growth by streptomycin. Some factors which affect the activity of streptomycin have also been investigated, and initial attempts to search for early biochemical changes during the period of inhibition of growth are described. Some of these findings have been published in abstract form (Hancock, 1960).

METHODS

Organism, growth and harvesting. Throughout this work *Staphylococcus aureus* strain Duncan (Gale & Taylor, 1947) was grown in the peptone-glucose medium (PG) described previously (Park & Hancock, 1960), adjusted accurately to a pH value of 7·2. In early experiments difficulties were encountered due to variations in the effects of streptomycin in different batches of media, which sometimes made comparisons between different experiments difficult. These variations occurred in spite of care in controlling conditions of sterilization and final pH value, and eventually all the experiments described here were carried out using medium drawn from two large batches, which were tested to ensure that the response of the organism to streptomycin was identical in each batch. In some experiments the composition of the medium was changed slightly: when radioactive glucose was used, the final glucose concentration in the growth medium was 0·02 % (w/v); when radioactive phosphate was used, the phosphate in the medium was partially replaced by 2-amino-

2-hydroxymethyl-propane-1:3 diol, as described by Park & Hancock (1960). These changes in the medium did not result in any change in the response of the organism to streptomycin.

For counting numbers of viable organisms, the normal PG medium, solidified by addition of 2 % (w/v) agar (New Zealand), was used.

All experiments were performed using cultures growing at 37° in Erlenmeyer flasks shaken in a constant-temperature water bath; the prewarmed medium was inoculated with 10 % (v/v) of an overnight culture grown in the same way. To grow organisms under anaerobic conditions, similar flasks were fitted with a bung with a tube and tap, through which the flask was twice evacuated and flushed with nitrogen. For experiments on the growth of cultures under low oxygen tensions, organisms were grown in test tubes (10 cm. × 1 cm.) which fitted the Unicam SP. 600 spectrophotometer; these tubes were incubated in an air incubator at 37°. The tubes contained 5 ml. of medium; before use they were heated in a boiling water bath for 5 min. to remove dissolved air, and a layer of sterile liquid paraffin was placed on the surface. Before measuring the optical density of such cultures, the organisms were resuspended by gentle pipetting with a Pasteur pipette, care being taken to introduce no air through the layer of paraffin.

The extent of growth was followed by determining the optical density of cultures at 700 mμ, using the Unicam SP. 600 spectrophotometer. When streptomycin was added to a portion of a culture, this was done when the optical density was between 0·30 and 0·40.

Organisms were harvested on 'Oxoid' membrane filters using either 6 cm. diameter filters in a 'Quickfit' all-glass assembly or 8 cm. diameter filters in an assembly made in this laboratory. The organisms were washed on the membrane filter with ice-cold water. In most cases they were then resuspended, either in ice-cold water for the preparation of cell suspensions for fractionation or metabolic experiments, or in prewarmed growth medium when subsequent growth was to be followed, by covering the filter with suspending medium and gently rubbing off the organisms with a fine glass rod.

Sensitivity tests and counting of viable organisms. The minimum inhibitory concentrations of streptomycin and related compounds were determined in tubes containing 5 ml. of medium; the tubes were inoculated with 1 drop of a shaken culture which had grown for 24 hr. and was in the stationary phase of growth, and were incubated for 16 hr. at 37°.

For counting numbers of viable organisms, successive tenfold dilutions were made of samples of a culture, using growth medium as diluent. Three replicate samples, taken from each of four successive dilutions, were placed on the surface of dried agar plates using a calibrated dropper according to Miles & Misra (1938). Colonies were counted after incubation of the plates at 37° for 24 hr. under aerobic conditions or after 48 hr. under anaerobic conditions. Colony counts were only recorded from those plates on which the samples had contained between 10 and 100 viable organisms. In the values presented here the extreme values always differed by less than 10 % from the mean value. The number of organisms in a culture which were resistant to streptomycin was determined similarly, by plating on agar containing streptomycin at the required concentration. In the experiments described in Fig. 2, diluted samples were spread over the whole surface of plates, using a glass spreader.

In determinations of numbers of viable organisms under the conditions used here, no difficulties were encountered due to irregular effects of dilution or to non-reproducibility in replicate samples, such as Barer (1951) found with *Bacterium lactis aerogenes*.

Fractionation of organisms. To prepare hot-water extracts of organisms, the membrane filter covered with organisms was rapidly transferred, usually within 3 min. of harvesting, to a beaker of boiling water and immersed for 10 min. The filter was then removed and rinsed with water; the aqueous extract was centrifuged at 3000 *g* for 15 min. to remove cell debris and the supernatant solution was freeze-dried. The cold trichloracetic acid (TCA)-soluble fraction was prepared by pipetting a sample of a suspension of organisms, washed off a membrane filter as described above, into an equal volume of 10 % (w/v) TCA. After 30 min. at 5°, the suspension was centrifuged at 3000 *g* for 15 min., the pellet was resuspended in 5 % (w/v) TCA and again centrifuged, and the supernatant solutions were combined. The compounds in this fraction were separated using the fractionation procedure A of Umbreit, Burris & Stauffer (1951). In one experiment the fractionation scheme used by Umbreit (1953) was used.

Samples (2–10 ml.) taken from cultures containing ^{14}C-amino acids, ^{14}C-glucose or ^{32}P-orthophosphate were pipetted rapidly into an equal volume of ice-cold 10 % (w/v) TCA; the cold-TCA-insoluble residue was then fractionated by the procedure of Park & Hancock (1960). Total radioactivity in the organisms was determined using organisms harvested rapidly on a 2 cm. diameter membrane filter; the filter was washed twice with 2 ml. quantities of ice-cold water and mounted on a planchette for radioactivity determinations. The difference between the total radioactivity and that in the cold-TCA-insoluble fraction gave the radioactivity in the cold-TCA-soluble fraction. The abbreviation cpm has been used for counts per minute.

With the exception of $Ba^{14}CO_3$, radioactive samples were mounted on planchettes under conditions where self absorption was less than 5 %, as described elsewhere (Park & Hancock, 1960). Precipitates of $Ba^{14}CO_3$ were suspended in 50 % (v/v) aqueous ethanol and pipetted on to similar planchettes. In experiments in which the production of $^{14}CO_2$ from 1-^{14}C, 6-^{14}C and generally-^{14}C-labelled glucose was measured in three identical cultures, it was assumed that the total amount of CO_2 evolved in each culture was the same. Only the ratios of the specific activities of the $^{14}CO_2$ produced in the three cultures were required, and not absolute values, and the further assumption was made that the weights of $Ba^{14}CO_3$ on each of the three planchettes from any single experiment were identical; corrections for self absorption were not made and the results of such experiments are expressed as ratios of the observed radioactivity on each planchette.

For estimations of nucleic acids, samples (10 ml.) of a culture were taken into perchloric acid (final concentration 0·25 N); subsequent treatment and estimation of deoxyribonucleic acid (DNA) were by the method of Burton (1956). Total nucleic acid was estimated by measuring absorption at 260 mμ, using the same extract as that used for DNA estimation. The results of nucleic acid estimations have been expressed in terms of amounts of nucleic acid phosphorus, using the conversion factor given by Burton (1956) for DNA and that of Gale & Folkes (1953) for total nucleic acid (TNA).

Manometric techniques. Warburg manometer flasks contained 300 μmoles K-phosphate buffer pH 7·2 and *c.* 5 mg. dry weight of organisms in a volume of 2·0 ml. 1·0 ml. of a solution (0·01 M) of the appropriate substrate was added from the side arm to start the reaction. The gas phase was air and the temperature 37°. To measure oxygen uptake, 0·2 ml. of 40 % (w/v) CO_2-free KOH was placed in the centre well; a folded filter paper was added, except when production of $^{14}CO_2$ was being measured. The rate of gas uptake was followed at 5 min. intervals for a 30 min. period, over which it was linear; experimental values were corrected for the rate of endogenous gas exchange in the absence of substrate. All determinations were carried out in duplicate. When $^{14}CO_2$ was to be collected, 0·5 ml. of 25 % (w/v) TCA was added from a second side bulb to terminate metabolism, and the flask was shaken for 1 hr. to liberate dissolved CO_2. The contents of the centre well were removed with a Pasteur pipette, and the well was rinsed with 0·2 ml. H_2O; the combined solutions were transferred to a centrifuge tube and $BaCO_3$ was precipitated by addition of 2 ml. of 1 % (w/v) barium acetate and 2 ml. of 95 % (v/v) ethanol. After centrifuging (3000 *g*, 30 min.) the $BaCO_3$ was washed by resuspension in 95 % (v/v) ethanol followed by a similar centrifugation.

All substrates used were of analytical reagent grade; solutions of acidic substrates were neutralized with KOH before use.

Streptomycin and related compounds. The following preparations were used in these experiments: streptomycin sulphate (Glaxo), batch 2736, 740 units/mg.; dihydrostreptomycin sulphate (Glaxo), Batch 5562, 745 units/mg.; mannosidostreptomycin sulphate (L 484249-1-17) and streptidine sulphate (L 496386-1-4), both from Merck, Sharpe and Dohme Research Laboratories, New Jersey, U.S.A.; and streptomycinic acid sulphate (The Distillers Co. (Biochemicals) Ltd, Speke, Liverpool).

Concentrations of these compounds are expressed in terms of weights of the sulphates. Solutions in distilled water were prepared freshly each week and stored in the frozen state; solutions of streptidine and streptomycinic acid were adjusted to pH 7·0 before use.

Isotopic compounds. Generally-^{14}C-labelled amino acids, 1-^{14}C, 6-^{14}C and generally-^{14}C-labelled glucose, and ^{32}P-orthophosphate, were obtained from the Radiochemical Centre, Amersham. ^{14}C-Glucose and ^{32}P-orthophosphate were added to the growth medium to give final specific activities of 0·025 $\mu c/\mu$mole and 0·1 $\mu c/\mu$mole, respectively, and ^{14}C-amino acids to give a final concentration of 0·2 μc/ml.

Amino acids were estimated by the procedure of Cocking & Yemm (1955), slightly modified as described by Park & Hancock (1960).

Inorganic phosphate was determined by the method of Fiske & SubbaRow (1925), using one-tenth of the recommended volumes of sample and reagents.

Dry weights of washed organisms were determined after drying duplicate samples for 8 hr. at 105° in an air oven.

RESULTS

Minimum inhibitory concentrations of streptomycin and related compounds. The minimum inhibitory concentrations of streptomycin and some related compounds against this strain of *Staphylococcus aureus*, determined by a conventional technique after incubation for 16 hr., are given in Fig. 1. Counts of colony-forming organisms in samples taken from those tubes which showed no growth indicated that, under these conditions, streptomycin has a bactericidal, and not only a bacteriostatic, effect (Table 1). Dihydrostreptomycin has the same activity as streptomycin in such a test, and mannosidostreptomycin is less active; such relative activities of these three derivatives are frequently found (Waksman, 1949).

Table 1. *Bactericidal action of streptomycin in cultures used to determine minimum inhibitory concentrations*

Samples taken from an experiment identical with that in Fig. 1.

Streptomycin sulphate concentration (μg./ml.)	Viable organisms/ml.	
	Initial	After 16 hr.
8	$2{\cdot}1 \times 10^5$	$1{\cdot}8 \times 10^5$
10	$2{\cdot}1 \times 10^5$	$2{\cdot}2 \times 10^3$
12	$2{\cdot}1 \times 10^5$	$< 1 \times 10^2$

When similar tubes were incubated in nitrogen in a McIntosh & Fildes jar, considerably higher concentrations of streptomycin were necessary to inhibit growth (Fig. 1).

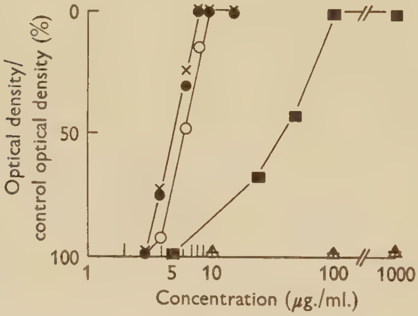


Fig. 1

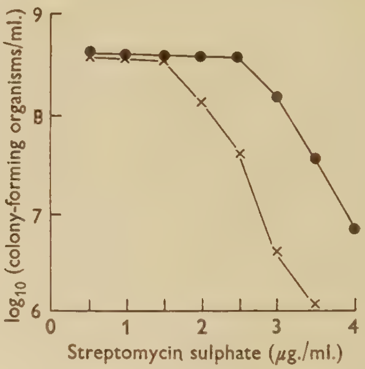


Fig. 2

Fig. 1. Inhibition of growth, expressed as optical density as % of control optical density, in cultures incubated for 16 hr. and containing streptomycin sulphate (●); dihydrostreptomycin sulphate (×); mannosidostreptomycin sulphate (○); streptidine sulphate (△); streptomycinic acid sulphate (+) (all aerobic); streptomycin sulphate (anaerobic) (■); at the indicated concentration.

Fig. 2. The numbers of colonies produced by identical samples taken from a growing culture, on plates containing streptomycin at the indicated concentration. Plates incubated aerobically (×) or anaerobically (●).

Distribution of sensitivity to streptomycin. In growing cultures of the type used for subsequent experiments, it was found that there was a considerable variation in the sensitivity of different organisms to streptomycin, as measured by the number of organisms able to form colonies on agar containing streptomycin at different concentrations (Fig. 2).

Effects of streptomycin on growing cultures. In initial experiments the effect of streptomycin on growth was followed by measurements of the optical density of cultures. It was found that the factor relating optical density to bacterial dry weight/ml. of culture is the same in streptomycin-inhibited organisms as in normal organisms, and since no major changes occur in the ratios of the major constituents of organisms following inhibition by streptomycin, optical density measurements were considered to be a reliable method of following the increase in cell constituents during inhibition of growth by streptomycin.

The effects of a range of concentrations of streptomycin on increase of optical density of growing cultures are shown in Fig. 3A; Fig. 3B shows the changes which occurred in the numbers of colony-forming organisms in the same cultures. In cultures growing anaerobically (under nitrogen), the rate of loss of viability was reduced; at lower concentrations of streptomycin this rate was almost zero and at higher concentrations it was still lower than the corresponding rate under aerobic conditions (Fig. 3B).

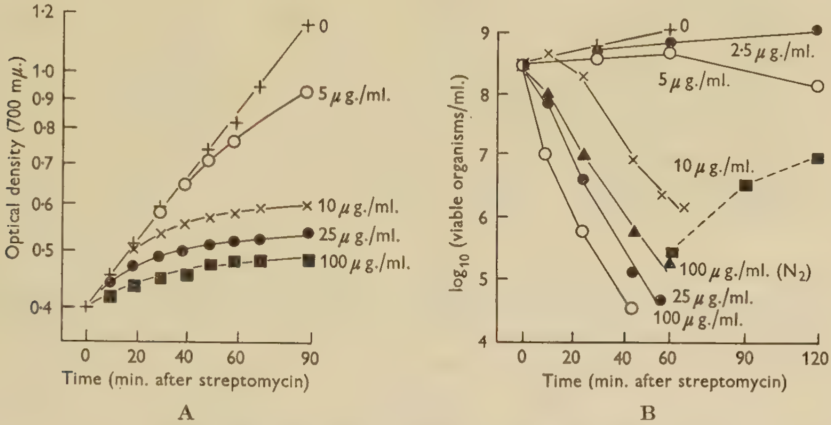


Fig. 3. Optical density and numbers of colony-forming organisms in growing cultures after addition of streptomycin sulphate to the indicated final concentration. Each concentration of streptomycin represents a separate experiment. A, Optical density; B, colony-forming organisms. The curve labelled (N_2) at 100 μg. streptomycin/ml. represents an experiment under anaerobic conditions. The points joined by dashed lines represent the number of organisms in the culture containing 10 μg. streptomycin sulphate/ml. which were able to produce colonies on plates containing 10 μg. streptomycin sulphate/ml., multiplied by 10^3.

If the initial pH of the growth medium was adjusted to values lower than that (7·2) used normally, the rate of development of inhibition of growth was less on addition of streptomycin (Fig. 4). The pH value of the medium normally used did not change during the exponential phase of growth.

Effects of streptomycin on non-growing organisms. If organisms were harvested from a growing culture when the optical density (700 mμ) reached 0·4, and resuspended at the same culture density in growth medium containing streptomycin sulphate (10 μg./ml.) for 75 min., no further growth occurred on resuspension in fresh growth medium, as would be anticipated as after this period of contact with streptomycin only *c.* 0·1 % of the organisms are viable (Fig. 3B). After this time there was a slow increase in optical density which may have been due to growth of a few streptomycin-resistant organisms, since there was an increase in the number of such organisms during this period (Fig. 3B). When, in similar experiments, the organisms were exposed to streptomycin for the same period either in growth medium cooled in an ice bath to 5°, or in medium which contained no nitrogen

Table 2. *Effect of conditions of exposure to streptomycin on rate of loss of viability*

Samples taken from a growing culture were incubated with streptomycin in an experiment similar to that in Fig. 5.

		Viable organisms/ml.			
			After 60 min. + streptomycin sulphate at (μg./ml.)		
Experiment	Conditions	Zero time	0	10	25
1	Growth medium, 37°, air	$2{\cdot}2\times10^9$	$6{\cdot}3\times10^9$	$2{\cdot}2\times10^6$	$2{\cdot}3\times10^5$
	Growth medium, 37°, nitrogen	$2{\cdot}2\times10^9$	$4{\cdot}8\times10^9$	$4{\cdot}2\times10^9$	$2{\cdot}0\times10^9$
	Growth medium, 5°, air	$2{\cdot}2\times10^9$	$2{\cdot}9\times10^9$	$2{\cdot}8\times10^9$	$2{\cdot}8\times10^9$
2	Growth medium, 37°, air	$1{\cdot}2\times10^9$	—	$9{\cdot}3\times10^7$	$1{\cdot}2\times10^5$
	Growth medium, 37°, air, + chloramphenicol (100 μg./ml.)	$1{\cdot}2\times10^9$	—	$9{\cdot}9\times10^8$	$4{\cdot}8\times10^8$
3	Growth medium without peptone, 37°, air	$5{\cdot}1\times10^8$	—	$4{\cdot}8\times10^8$	$5{\cdot}7\times10^8$

source, growth was immediately resumed on resuspension in fresh growth medium (Fig. 5). The numbers of viable organisms found after exposure to streptomycin under these various conditions are shown in Table 2. It was also found (Table 2) that if chloramphenicol (100 μg./ml.) is added to growing organisms simultaneously with streptomycin, loss of viability is prevented. Under these conditions, therefore, it appears that streptomycin is not bactericidal to *Staphylococcus aureus* in the absence of some process associated with growth of the organisms.

Reversal of action of streptomycin by streptomycin derivatives. Although streptidine sulphate is without detectable growth-inhibiting effect (Fig. 1), it was found that, at high concentrations relative to streptomycin, this compound can partially reverse the effect of streptomycin on growth (Fig. 6). This effect was not due to the addition of sulphate ions, since addition of the same quantity of $(NH_4)_2SO_4$ did not affect the activity of streptomycin. In similar experiments streptomycinic acid sulphate, at concentrations up to 1 mg./ml., produced only a very small reversal of the inhibition of growth by streptomycin sulphate (25 μg./ml.).

Synthesis of cell constituents during inhibition of growth by streptomycin. In the following experiments, cultures were divided when the optical density reached 0·3–0·4, and streptomycin was added to one portion; radioactive compounds, when used were added to the culture at the time of inoculation.

Figure 7 A shows progress curves of the incorporation of ^{14}C-L-glutamic acid into the major cell fractions. To facilitate comparisons of the subsequent increases, the curves have been adjusted to coincide at the time of addition of streptomycin. A similar experiment using ^{14}C-L-alanine, and in which incorporation of radioactivity into the hot-TCA-soluble fraction was used as a measure of synthesis of teichoic acid, as suggested by Park & Hancock (1960), is shown in Fig. 7B. It appears from these experiments that the rates of incorporation of these precursors into all the major cell fractions are reduced in a parallel manner during the period of development of growth inhibition by streptomycin.

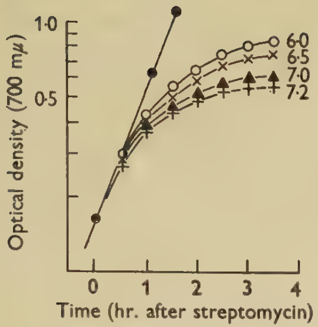


Fig. 4

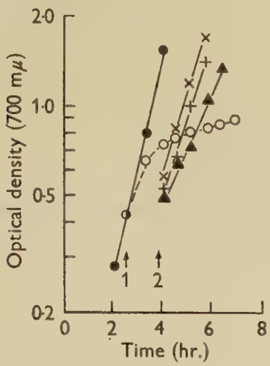


Fig. 5

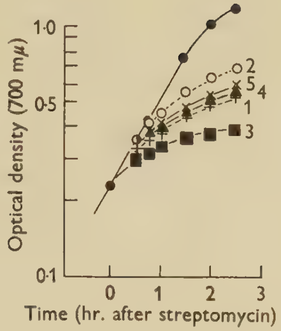


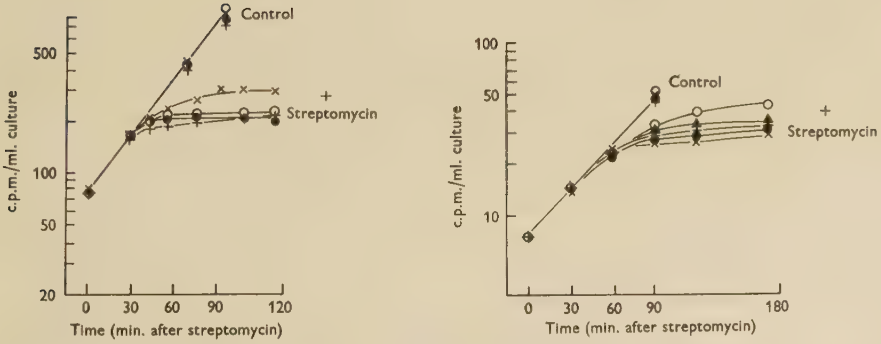
Fig. 6

Fig. 4. Optical density changes after addition of streptomycin sulphate (10 μg./ml.) to cultures growing in media adjusted to the indicated initial pH values; (●) control.

Fig. 5. Optical density changes after resuspension of streptomycin-treated organisms in fresh growth medium. At 1, the organisms were harvested and suspended in the following medium with addition of streptomycin sulphate (10 μg./ml.): (○), growth medium, 37°, in air; (×), as 1 but in nitrogen; (+), as 1 but at 5°; (▲), growth medium without peptone, 37°, in air. After 75 min., at 2, the organisms were filtered and resuspended in fresh growth medium at 37°. Controls (●).

Fig. 6. The effect of simultaneous addition of streptidine sulphate on the optical density changes following addition of streptomycin sulphate. Controls (●). The final concentrations (μg./ml.) were

Curve	Streptomycin sulphate	Streptidine sulphate
1	25	0
2	25	250
3	50	0
4	50	250
5	50	500



A

B

Fig. 7. Incorporation of ^{14}C-L-glutamic acid (A) and ^{14}C-L-alanine (B) into some major cell fractions after addition of streptomycin sulphate (10 μg./ml.). The values have been adjusted to coincide at the time of addition of streptomycin; the units on the ordinate represent the observed radioactivities multiplied by the following factors: Cold-TCA soluble fraction (●): A, ×1·83; B, ×2·38. Ethanol soluble fraction (×): A, ×75·0; B, ×5·90. Hot-TCA soluble fraction (▲): B, ×1·89. Protein (+): A, ×2·63; B, ×0·71. Cell-wall mucopeptide (○): A, ×10·0; B, ×1·0.

Determinations of the nucleic acid content of a culture after addition of streptomycin (Fig. 8) showed that the rates of synthesis of both TNA and DNA are also reduced simultaneously with the rate of incorporation of ^{14}C-L-proline into the protein fraction.

Table 3. *Quantities of some compounds in hot-water extracts from normal and streptomycin-inhibited organisms*

Values in parentheses are from a separate experiment.

	Total amino acids (μmole/g. cells)	Optical density (260 mμ)/g. cells	Phosphate (μmole/g. cells)	
			Inorganic	Total
Control organisms	451 (476)	1380 (1280)	171	625
Organisms grown with streptomycin sulphate (25 μg./ml.) for 60 min.	518 (512)	1220 (1350)	182	582

There appears therefore to be no specific or differential inhibition by streptomycin of the synthesis of any major constituent of growing organisms, such as occurs with the antibiotics chloramphenicol (Harrington, 1958; Mandelstam & Rogers, 1958) and with penicillin (Park, 1958).

The experiments using ^{14}C-amino acids (Figs. 7A and B) also suggest that there is no accumulation of amino acids in the metabolic pool during the period of inhibition of growth. This finding was confirmed by direct determinations of the total amino acid pool (Table 3). It was also found (Table 3) that during this time there were no large changes in the amounts of 260 mμ-absorbing substances or of inorganic phosphate in the metabolic pool. Separation of the pool amino acids by paper chromatography showed no detectable alterations in the relative amounts of each amino acid in the pool. These results suggest that the rates of synthesis, or of uptake into the organisms, of compounds in the metabolic pool decrease simultaneously with the rates of incorporation of these compounds into cell material.

Effect of streptomycin on incorporation of ^{32}P. Figure 9 shows progress curves of incorporation of radioactivity into the major fractions of the cold-TCA-soluble fraction, and into the cold-TCA-insoluble fraction, of organisms growing in the presence and absence of streptomycin, when ^{32}P-orthophosphate was added at the same time as streptomycin. In one similar experiment, organisms were harvested after 60 min. and the cold-TCA-soluble fraction was fractionated by the procedure used by Umbreit (1953); Table 4 shows the radioactivity found in the fractions obtained by this procedure.

Glucose metabolism by organisms grown in the presence of streptomycin. The foregoing experiments suggested that none of the major biosynthetic processes which were investigated are specifically inhibited during the period in which growth becomes inhibited by streptomycin. It seemed that these findings would be compatible with the existence of an early effect of streptomycin on reactions concerned with supply of metabolic energy. Since the breakdown of glucose is the major source of metabolic energy under the conditions of growth used here, attention was directed to the metabolism of glucose during the period of inhibition of growth by streptomycin. It was found that organisms harvested from cultures growing in the presence of streptomycin had significantly lower Q_{O_2} values than organisms from parallel control cultures. Figure 10A shows Q_{O_2}(glucose) values of organisms harvested at successive times following addition of streptomycin; the increase in optical density, and incorporation of ^{14}C-L-proline into protein, in the same cultures are included in Fig. 10A for comparison. The decrease in Q_{O_2} (glucose) value was apparently not due solely to a dilution of the population of normal organisms by organisms with impaired respiration, since at higher concentrations of streptomycin the Q_{O_2} value also decreased when expressed relative to a unit volume of culture (Fig. 10B). Some variation was found from time to time in the magnitude of the

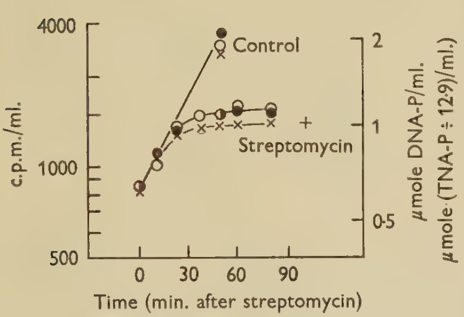


Fig. 8

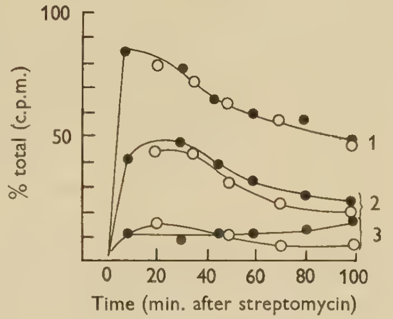


Fig. 9

Fig. 8. Synthesis of total nucleic acid (TNA) (○) and deoxyribonucleic acid (DNA) (●); and incorporation of ^{14}C-L-proline into protein (×), after addition of streptomycin sulphate (10 μg./ml.).

Fig. 9. Incorporation of ^{32}P-orthophosphate into some subfractions of the cold-TCA soluble fraction in a control culture (○) and after addition of streptomycin sulphate (10 μg./ml.) (●), expressed as % of the total radioactivity in the cells. Curve 1: total cold-TCA soluble fraction. Curve 2: barium-insoluble fraction. Curve 3: barium-soluble, ethanol-insoluble fraction.

Table 4. *Effect of streptomycin on incorporation of* ^{32}P *into some components of the cold-TCA soluble fraction*

		Radioactivity (counts/min.) in			
	Increase in dry weight (mg./ml./hr.)	Intact organisms	Cold-TCA soluble fraction	Cold-TCA soluble fraction (organic phosphates)	Fraction containing 'new compound' of Umbreit (1953) (organic phosphates)
Control organisms (C)	0·50	128,700	99,980	68,700	1,225
Organisms grown with streptomycin sulphate (25 μg./ml.) for 60 min. (S)	0·34	96,800	70,370	48,400	990
S, % C	68·0	75·4	70·4	70·5	80·9

changes in Q_{O_2} (glucose) following addition of streptomycin; Table 5 shows results from two experiments to indicate the range of this variation. It is clear from Fig. 10 A, however, that a decrease in Q_{O_2} value occurs before the rate of synthesis of cell material begins to decrease, and that the depression of Q_{O_2} value is considerable by the time that inhibition of growth becomes complete.

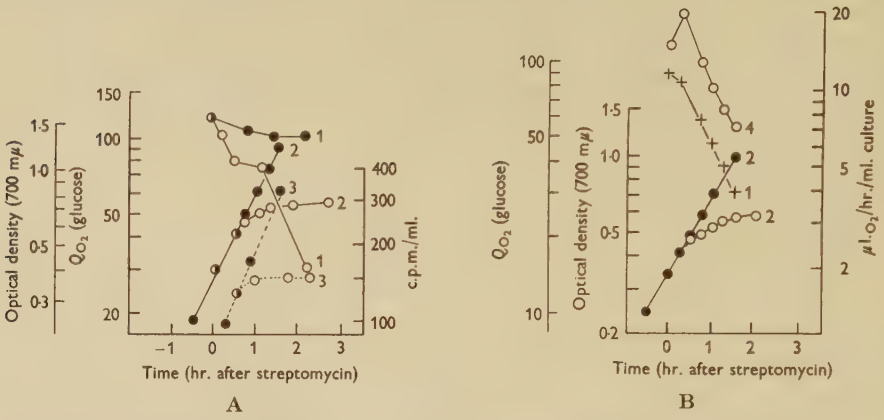


Fig. 10. Q_{O_2} (glucose) values (curves 1) of organisms harvested from control cultures (●) and from cultures after addition of streptomycin (○). A. Streptomycin sulphate 25 μg./ml. Values of the optical density of the cultures (curves 2), and of the incorporation of ^{14}C-L-proline into the protein fraction (curves 3) are included for comparison. B. Streptomycin sulphate concentration 50 μg./ml. Curve 1 (Q_{O_2} glucose) from culture after the addition of streptomycin (+). Curve 4 represents the rate of glucose oxidation of harvested organisms expressed relative to a unit volume of culture.

To investigate the metabolism of glucose by routes other than the Embden–Meyerhof pathway, the production of $^{14}CO_2$ from 1-^{14}C-glucose (1-CO_2), 6-^{14}C-glucose (6-CO_2) and generally-^{14}C-labelled-glucose (CO_2) was investigated in normal organisms and organisms growing in the presence of streptomycin, with the results shown in Table 6. The interpretation of experiments of this type has been discussed by Heath & Koffler (1956), who have shown how the ratio of the quantities of 1-CO_2 to 6-CO_2 can be related to the proportion of the total glucose oxidized which is metabolized via pathways in which C_1 of glucose is preferentially oxidized, such as the pentose phosphate cycle. In the presence of streptomycin there appears to

Table 5. Q_{O_2} *(glucose) of organisms after growth in the presence of streptomycin at varying concentrations*

Results of two experiments (30 min and 60 min) chosen to show the variation observed in the magnitude of the reduction of Q_{O_2} values in streptomycin-inhibited organisms.

Concentration of streptomycin sulphate (μg./ml.)	Q_{O_2} (glucose) of streptomycin-inhibited organisms, as % of that of control organisms, after growth in the presence of streptomycin for	
	30 min.	60 min.
10	64	92
20	42	69
40	40	49
60	35	45
80	29	39

be no significant difference in the very small production of 6-CO_2 (which may indicate a very low level of cyclic operation of the tricarboxylic acid cycle in this organism), or in the proportion of glucose metabolized via a C_1-specific oxidation.

Table 6. *Production of* $^{14}CO_2$ *from generally- and specifically-*^{14}*C-labelled glucoses during growth in presence and absence of streptomycin*

Cultures growing in manometer flasks; the specific activity of ^{14}C-glucose was the same in each culture.

		Radioactivity (counts/min.) in CO_2, as % of that in CO_2 from generally-^{14}C-labelled glucose, produced from			
		1-^{14}C-glucose		6-^{14}C-glucose	
Experiment	Sample time (min.)	Control culture	+ Streptomycin sulphate (25 μg./ml.)	Control culture	+ Streptomycin sulphate (25 μg./ml.)
1	30	22·2	18·2	1·3	0·9
	60	14·3	14·5	0·9	0·7
2	15	15·5	14·2	0·9	0·8
	45	15·5	17·0	1·0	0·8

Oxidation of other substrates by organisms grown in the presence of streptomycin. It was of interest to investigate if organisms grown in the presence of streptomycin have a lower oxidative activity towards other substrates as well as towards glucose. The only substrates which this strain of *Staphylococcus aureus* was found to be capable of oxidizing at significant rates were glucose, lactate, glycerol, and formate. Q_{O_2} values obtained in two experiments for oxidation of these substrates by organisms harvested after growth in the presence of streptomycin and by organisms grown in parallel control cultures are given in Table 7, which also includes similar results using dihydrostreptomycin. In these experiments the organisms were harvested when growth inhibition was first detectable. The addition of streptomycin

Table 7. Q_{O_2} *values of normal and streptomycin-inhibited organisms*

	Q_{O_2} value of			
			Normal organisms + streptomycin sulphate at	
Substrate	Normal organisms	Organisms grown with streptomycin sulphate (25 μg./ml.) for 45 min.	25 μg./ml.	100 μg./ml.
Glucose	73·5	55·2	84·1	83·2
	93·0	74·1	—	—
Lactate	19·7	13·8	—	—
	14·2	8·7	—	—
Glycerol	48·0	9·8	49·2	50·5
Formate	27·0	10·1	22·2	18·2
	14·7	0·9	—	—
Glucose	92·5	62·5 (dihydrostreptomycin sulphate)	—	—

sulphate, at concentrations up to 1 mg./ml., to the contents of the manometer flask resulted in no depression of the Q_{O_2} values of normal organisms.

Specificity of reduction of oxidation rates. It was important to determine if the reduced Q_{O_2} values of streptomycin-inhibited organisms were due to a specific effect of streptomycin, or were merely a consequence of reduction in the growth rate. Q_{O_2} values of organisms whose growth had been inhibited by other antibiotics, or by cooling, were therefore determined, with the results shown in Table 8. The growth rates of the cultures in these experiments were reduced to zero when the organisms were harvested, with the exception of the cultures containing chloramphenicol and aureomycin. In these cultures, increases in optical density and in dry weight of organisms occur due to synthesis of cell-wall material (Hancock & Park, 1958; Park, 1958), but since there is no increase in cellular protein the Q_{O_2} values for these cultures were calculated relative to the dry weight of organisms at the time of addition of the antibiotics. The reduction in Q_{O_2} values appears to be specific to organisms whose growth has been inhibited by streptomycin.

Table 8. *Effect of inhibition of growth on Q_{O_2} values*

Organisms harvested 60 min. after addition of antibiotics or after transfer of the culture to the refrigerator.

	Q_{O_2} values of organisms on	
Growth of culture inhibited by	Glucose	Formate
— (control)	104·8	51·6
Penicillin (10 units/ml.)	102·2	42·5
Chloramphenicol (50 μg./ml.)	100·8	42·5
Aureomycin hydrochloride (10 μg./ml.)	103·2	51·0
Streptomycin sulphate (25 μg./ml.)	68·5	18·4
Cooling	122·2	52·0

DISCUSSION

Although the experiments reported here on factors affecting the activity of streptomycin were not intended to be comprehensive or exhaustive, but only to establish a basis for biochemical work, they show a number of characteristics of the bactericidal action of this antibiotic on *Staphylococcus aureus* which are important in considering possible modes of action of streptomycin. It has been frequently demonstrated (for review see Lightbown, 1957) that, as found here, the activity of streptomycin against facultatively anaerobic organisms is less when growth occurs anaerobically, and this may be related to the very low activity of streptomycin against many obligatory anaerobic organisms. We are not aware of any previously published observations which show that the bactericidal action of streptomycin on growing organisms can be prevented if growth is arrested during contact with streptomycin, as appears to be the case with *S. aureus*; however, while this paper was in preparation, Anand & Davis (1960) reported that streptomycin is not bactericidal to *Escherichia coli* in the presence of chloramphenicol. In some instances in which organisms lose viability only under conditions in which growth can occur, it has been shown that 'unbalanced synthesis' of some component of the organism may be responsible (for review see Roberts, McQuillen & Roberts, 1959). If such a process occurs in *S. aureus* in the presence of streptomycin, it does not apparently involve 'unbalanced synthesis' of any of the major cell constituents which have been investigated here.

The concentrations of streptomycin which have been used in these experiments are higher than the minimum inhibitory concentration determined by a conventional test (Table 1). The results in Fig. 3A show that no effect of streptomycin at concentrations below 5 μg. streptomycin sulphate/ml. can be observed on growth during the exponential phase under the conditions used here; nevertheless, the organisms do lose viability at these low concentrations of streptomycin but at a very low rate (Fig. 3B). It was convenient to carry out biochemical experiments over a reasonably short period of time, and the concentration of streptomycin used here was chosen to enable this to be done while still allowing a reasonable period in which to search for biochemical changes before growth became inhibited. The observed time lag which occurs before inhibition of growth becomes complete could be due to a number of possible factors, including the period taken for streptomycin to reach its site of action, or the time taken to utilize preformed precursors in a metabolic pathway blocked by streptomycin.

Although no large changes were found in the distribution of ^{32}P in the major fractions of the cold-TCA-soluble fraction in organisms grown in the presence of streptomycin, these results, together with those showing no change in the total amount of 260 mμ-absorbing material in this fraction, are regarded as only preliminary attempts to investigate any changes in these fractions. The possibility remains that redistributions occur in the individual components in these fractions although the total quantities are unchanged, and it is difficult to draw conclusions until more detailed results are obtained. The ^{32}P-labelled compounds which occur in the fractions obtained from *Staphylococcus aureus* using the fractionation procedure of Umbreit (1953) have not been identified, but no inhibition by streptomycin could be detected of incorporation of ^{32}P into that fraction into which such incorporation is inhibited by streptomycin in *Escherichia coli* (Umbreit, 1953). It is possible that inhibition of incorporation of ^{32}P into a minor component of this fraction would be difficult to detect if large amounts of other compounds were present. Similarly, although there is no large change in the total quantity of 260 mμ-absorbing compounds in hot-water extracts of organisms grown in the presence of streptomycin, changes in the quantities of individual compounds in this fraction have been detected in preliminary separations by ion-exchange chromatography using the procedure of Hurlbert, Schmitz, Brumm & Potter (1954). We have found no evidence of leakage of compounds from the metabolic pool of organisms growing in the presence of streptomycin, as has been reported to occur in *E. coli* (Anand & Davis, 1960).

The earliest biochemical change which we have been able to detect is the reduced ability of organisms grown in the presence of streptomycin to oxidize glucose and other substrates. Jackson (1958) has described experiments similar in some respects to those reported here, in which growing cultures of *Staphylococcus aureus* were exposed to dihydrostreptomycin. In this case it was also found that organisms harvested from such cultures have lower rates of oxidation of glucose and succinate than normal organisms; succinate oxidation was found to be depressed to a greater extent than glucose oxidation. Oxidation of glucose and pyruvate by cultures of

Shigella sonnei is also reduced when streptomycin, at concentrations which inhibit increase in cell material, is present in the growth medium (Henry, Housewright & Berkman, 1949).

It would be anticipated that a reduced ability to oxidize glucose would be reflected as a reduction in the rate of metabolism of glucose by organisms during growth in the presence of streptomycin. Experiments in which the pathways of glucose metabolism by this strain of *Staphylococcus aureus* have been investigated, using specifically-^{14}C-labelled glucoses, will be described in a further paper, but some observations on the effect of streptomycin are of interest. ^{14}C-Glucose was added to a culture at the same time as streptomycin, and hot-water extracts were prepared from the organisms as soon as inhibition of growth could be detected; the radioactive compounds in these extracts were separated by paper chromatography. The differences observed between control and streptomycin-treated organisms showed that, in the presence of streptomycin, relatively more radioactivity occurred in intermediates of the Embden–Meyerhof pathway, and relatively less in metabolic end-products, indicating that there is a reduction in the rate at which glucose is metabolized via this pathway. However, if organisms grown in the presence of streptomycin were allowed to metabolize ^{14}C-glucose to completion, the final distribution of ^{14}C among the products did not differ from that produced by normal organisms.

The reduced Q_{O_2} values of streptomycin-inhibited organisms do not appear to be a consequence of inhibition of growth, in so far as they are not observed in organisms whose growth has been inhibited by other means. When inhibition of growth by streptomycin is first detectable, the reduction in rate of glucose oxidation is not large, and the possibility that it is a cause of growth inhibition must remain open; it is possible that these early changes are consequences of yet an earlier effect of streptomycin. However, there does appear to be some correlation between the growth rate at any time after addition of streptomycin and the Q_{O_2} (glucose) values of organisms harvested at that time. The reductions in Q_{O_2} values for other substrates are greater than that for glucose, and these changes suggest that in organisms grown in the presence of streptomycin there could be a metabolic block in some reaction common to the metabolism of all these substrates. Since the Q_{O_2} value for formate oxidation is reduced in streptomycin-inhibited organisms, and since the tricarboxylic acid cycle operates only to a very small extent in this organism (as shown by the rate of production of $^{14}CO_2$ from 6-^{14}C-glucose), it is possible that this block is in the chain of respiratory enzymes; this possibility is being investigated further.

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Species Differentiation of Human Vaginal Lactobacilli

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SUMMARY

The designation 'Döderlein's bacillus' for human vaginal strains of the genus *Lactobacillus* is vague and unsatisfactory. Thirty-five strains of lactobacilli from twenty-one normal non-pregnant women aged 21–40 were isolated; *c.* 25 % were heterofermentative. Of 21 isolates studied in detail, 14 were *L. acidophilus* and two were *L. casei* var. *rhamnosus* (Rogosa *et al.* 1953). The heterofermentative species comprised four strains of *L. fermenti* and one of *L. cellobiosus* (Rogosa *et al.* 1953). Glycogen fermentation, which was found only in certain strains of *L. acidophilus*, was a variable characteristic. In addition, *Leuconostoc mesenteroides*, which on examination of smears can be confused with short rod forms, was also isolated.

INTRODUCTION

At birth the human vagina is sterile and the vaginal secretion is at pH 5·0 or less (Miura, 1928; Hardy, 1941; Cruickshank & Sharman, 1934*a*, *b*). It has been assumed that bacterial implantation takes place within a few hours and for 2 or 3 weeks postnatally 'a simple homogeneous flora of Gram-positive bacilli, known as Döderlein's vaginal bacillus, is established in the vagina in association with a slightly acid non-purulent secretion' (Cruickshank & Sharman, 1934*b*). Shortly after this the flora changes and until puberty normally comprises micrococci, streptococci, diphtheroids, but seldom 'Döderlein's bacillus' (Döderlein, 1892). At puberty and before menstruation, the flora again changes to one predominantly of lactobacilli, and remains so in normal individuals throughout the reproductive period. At menopause there is a final change to the mixed flora found before puberty (Pettit & Hitchcock, 1933; Soeken, 1926; Dychno & Dertschinsky, 1930). These changes are thought to be correlated with the appearance of glycogen in the mucosal epithelium induced by oestrin formation (Miura, 1928; Cruickshank & Cruickshank, 1931; Cruickshank & Sharman, 1934*a*, *b*). Döderlein's (1892) original thesis, also maintained by others, was that the production of lactic acid by the Gram-positive bacilli served as one of the protective measures against infection.

Organisms assumed to be 'Döderlein's bacillus' have been variously designated as *Bacillus vaginae*, *B. vaginalis longus*, *B. vaginalis*, *Acidobacterium doederlinii*, *Plocamobacterium vaginae*, *Lactobacillus doederlein*, *Bacillus lacticus* and *Lactobacillus* *acidophilus* (*Bergey's Manual*, 1948). The Gram-positive rods isolated in previous work are so incompletely described that it is impossible to know what they really were, except that they may often have been lactobacilli, or even diphtheroids. In a number of investigations diagnoses for 'Döderlein's bacillus' have been made from studies of smears only.

During an investigation at the U.S. National Institutes of Health by one of us (M.R.), the purpose of which was to explore the relation between bacterial and trichomonad metabolism, modern methods of study (Rogosa *et al.* 1953; Sharpe, 1955) were used to isolate and characterize vaginal lactobacilli from normal non-pregnant women. It was almost immediately apparent that the isolated strains did not form a homogeneous group, and certainly not within a species, since homofermentative and heterofermentative organisms were obtained.

METHODS

The vaginal secretions were obtained by the swab technique designed to minimize external contamination (Miura, 1928; Soeken, 1926; Cruickshank & Sharman, 1934*a*, *b*). The samples were plated on the selective lactobacillus medium of Rogosa, Mitchell & Wiseman (1951*a*, *b*) and incubated at 37° anaerobically for 4 days in an atmosphere of 90 % (v/v) N_2 and 10 % (v/v) CO_2. Isolations from representative colonies were made and replated as required to ensure purity.

The methods used for the identification of the isolates combined cultural, biochemical and serological techniques to establish correlations of characteristics. These methods have previously proved to be highly successful in characterizing lactobacilli from a variety of sources (Rogosa *et al.* 1953; Sharpe, 1955) and have been developed from the basic studies of Orla-Jensen (1919, 1943).

RESULTS

Thirty-five strains of lactobacilli from twenty-one individuals, aged 21–40 years, were isolated; 21 of these isolates were studied in detail to determine their species designation. The remaining 14 strains were lost, but not before tests for gas production had been completed. Heterofermentative lactobacilli for the total sampling were found in approximately the ratio of 1:4 as shown in Table 1.

Fourteen of the 21 isolates were typically *Lactobacillus acidophilus*, and were so designated by means of the criteria of Orla-Jensen (1919, 1943), Rogosa *et al.* (1953), Sharpe & Mattick (1957), Harrison & Hansen (1950), de Man (1956), Wheater (1955), and others. Serological techniques for the confirmatory identification of *L. acidophilus* are not available since successful group antisera for either the agglutination or precipitin techniques have not yet been produced. The two strains of the second homofermentative species, *Lactobacillus casei* var. *rhamnosus* (Rogosa *et al.* 1953; Sharpe & Wheater, 1957) were typical in all respects, including possession of the group C antigen.

The remaining isolates were heterofermentative. Two of the strains of *Lactobacillus fermenti* had typical characteristics, including the group F antigen. However, two of the strains, although typical in other respects, did not react serologically and thus resembled many of the strains of intestinal origin.

In a previous examination by one of us (M. E. S.) of the faeces of twelve breast-fed infants 6–11 days old, in addition to the so-called *Lactobacillus bifidus* (Sundman, Björksten & Gyllenberg, 1959), authentic lactobacilli were also isolated from three

Table 1. *Species characteristics and frequency distribution of human vaginal lactobacilli*

	No. of strains	Gas from glucose	NH_3 from arginine	Configuration of lactic acid	Aesculin cleavage	Amygdalin	Arabinose	Cellobiose	Glycogen	Mannitol	Mannose	Melezitose	Melibiose	Raffinose	Rhamnose	Salicin	Sorbitol	Sorbose	Trehalose	Xylose
L. acidophilus	14	−	−	DL	+	+	−	+	±	−	+	−	−	±	−	+	−	−	+	−
L. casei var. *rhamnosus*	2	−	−	L(+)	+	+	−	+	±	+	+	+	−	−	+	+	+	+	+	−
L. fermenti	4	+	+	DL	−	−	±	−	−	−	−	−	+	+	−	−	−	−	−	±
L. cellobiosus	1	+	+	DL	+	+	−	+	−	−	+	−	+	+	−	+	−	−	+	−

± = variable reaction. All strains fermented glucose, galactose, lactose, fructose and maltose. Half the strains of *L. acidophilus* and both strains of *L. casei* var. *rhamnosus* fermented dextrin slowly or slightly. All except one strain of *L. casei* var. *rhamnosus* fermented sucrose. Since the sucrose fermentation is adaptive for this organism, this is not uncommon. No strains fermented adonitol, erythritol, inulin, α-methyl D-mannoside. α-methyl D-glucoside was fermented by one strain of *L. casei* var. *rhamnosus* only.

The nutritional characteristics (not shown) were identical with typical species behaviour as described by Rogosa *et al.* (1953).

of the infants; these strains were all *L. fermenti*, giving the typical biochemical and serological reactions of this species. Witkowski (1935) showed that the mouth of the infant shortly after birth contains organisms in proportions corresponding closely to the flora of the mother's vagina. 'The intestine at birth is either sterile or contains a few bacteria; infection rapidly occurs, partly from above and partly from below' (*Topley and Wilson's Principles*, 1955). The strains of *L. fermenti* described here may also have had a similar origin. The heterofermentative strain of *L. cellobiosus*, a species previously isolated from the mouth, had the modal characteristics described by Rogosa *et al.* (1953).

Occasionally certain *Leuconostoc* species may be isolated from the medium designed for the selection of lactobacilli (Perry & Sharpe, 1960). It is of incidental interest that *L. mesenteroides* was isolated in the present work and found to be identical with the organism as described by Garvie (1960). This appears to be the first reported isolation of this heterofermentative lactic acid coccus from the human vagina. It is also of interest since in morphology this organism often resembles the short coccobacillary forms of lactobacilli, particularly in acidic environments. Thus examinations of smears for 'Döderlein's bacillus' might be misleading in underestimating the real coccal population.

DISCUSSION

Except that Döderlein (1892) had most probably isolated a lactobacillus, the species identities of the vaginal lactobacilli have not previously been satisfactorily established. In many instances any rod-like bacterium was identified as 'Döderlein's bacillus' on the basis of morphology alone. Although Miura (1928) had identified the lactic acid produced by his isolate as the DL enantiomorph, it is impossible to identify the organism from the fragmentary data. Many species of lactobacilli produce DL-lactic acid. Thomas (1928), who used inadequate and somewhat questionable tests, concluded that 'Döderlein's bacillus' was *Lactobacillus acidophilus*, but recognized that this terminology was loose and generic, as was the case for the wide spectrum of oral lactobacillus species also previously designated as *L. acidophilus* (Rogosa *et al.* 1953; Tilden & Svec, 1950).

Although Miura (1928) found that his isolate did not ferment glycogen, Wheater (1955) observed that 43 % of her strains of *Lactobacillus acidophilus* did so. We have recently re-studied representative strains of Wheater's collection and have confirmed her results. On originally testing our isolates, none of our strains fermented glycogen. However, it was noted that the initial pH value of the medium was relatively high for lactobacilli (pH 7·0). When the tests were repeated with 9 representative strains at more favourable pH values (5·0, 5·8, 6·5) five of them fermented glycogen, but usually weakly. This behaviour was not associated with consistent species activity but rather with individual strains and in repeated experiments seemed to be an adaptive and variable phenomenon, independent of anaerobiosis or aerobiosis. Thus it seems highly probable that these lactic acid bacteria obtain available carbohydrate from the enzymic conversion of glycogen if not other carbohydrates, by the tissues or by micro-organisms other than lactobacilli. Also a considerable heterofermentation in the vagina is strongly suggested by the present results.

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Further Mutational Studies on the PAS Resistance of *Mycobacterium tuberculosis* var. *hominis*

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SUMMARY

Mutational studies on the PAS (sodium *p*-aminosalicylate) resistance of *Mycobacterium tuberculosis* var. *hominis*, strain H37RV have been carried out. It has been assumed that one type of population structure of clones with relation to drug resistance corresponds to one genotype. Clones derived from single colonies obtained on different PAS concentrations produced three types of population structure; the first type is similar to the population structure of the parent strain which has not been previously exposed to the drug and its degree of resistance is up to 0·2 μg. PAS/ml. The second and the third types are different from the parent strain and their degrees of resistance are up to 3 and 1000 μg. PAS/ml., respectively. Neither intermediate population structures nor intermediate degrees of resistance were clearly observed. From these results, it may be concluded that there are only two genotypes responsible for PAS resistance, one determining a lower, and the other a higher degree of resistance.

INTRODUCTION

Szybalski & Bryson (1953) reported from their studies of survival curves of *Bacillus megaterium* to PAS (*p*-aminosalicylate) that resistance is a single step pattern. However, genetic information on the PAS resistance of micro-organisms is still scanty (Bryson & Szybalski, 1955) and the purpose of this paper is to study the PAS resistance of *Mycobacterium tuberculosis* from the mutational aspect.

METHODS

Strain. Mycobacterium tuberculosis var. *hominis*, strain H37 Rv, was used throughout. The parent strain was derived from a single colony.

Medium. Ogawa's egg medium was prepared as follows: basal solution (1 % KH_2PO_4 and 1 % sodium glutamate), 100 ml.; whole eggs, 200 ml.; glycerol, 6 ml.; 2 % aqueous solution of malachite green, 6 ml. The medium was poured in 8 ml. amounts into tubes, 170 × 17 to 18 mm., sloped, and sterilized at 90° for 60 min. PAS sodium *p*-aminosalicylate (Shionogi Co.) was dissolved in distilled water and added to the medium before sterilization.

Population structure of the clone. As the characters of a single organism cannot be observed, those in relation to drug resistance may be expressed as the population structure of a clone. The present study was conducted by assuming that one type of population structure (character) of a clone represents one phenotype, and probably corresponds to one genotype. Colony size on a given drug concentration cannot be used in this instance as a measure of the character (the degree of resistance), since colony size is affected considerably by experimental conditions. There is, for example, a variation in colony size depending upon the number of colonies per unit area of medium, even among those colonies derived from cells belonging to the same clone and having the same genotype. Thus, in order to obtain data independent of such variation, the degree of resistance was expressed as the population structure of a clone, which was measured by its survival ratios on different concentrations of PAS.

Method of determining the survival ratio. A single colony was picked and subcultured on media without drug for 6 weeks. The resulting culture was suspended in saline after shaking with glass beads for 10 min. Tenfold dilutions were made from this suspension, and 0·02 ml. of each dilution inoculated on to a set of media containing graded concentrations of PAS and to control media without PAS. The inoculation was made by a whirl loop delivering 0·02 ml. (Tsukamura & Noda, 1957). Counts were made after 4 weeks of incubation, and the number of surviving colonies was determined as an average of ten replicates.

RESULTS

Population structure of the parent strain not exposed to PAS

The results on the population structure of the parent strain, expressed as survival ratios on various concentrations of PAS, are shown in Table 1. It will be noted that the survival ratio dropped discontinuously at a concentration between 0·1 and 0·2 μg. PAS/ml., and decreased slowly between 0·2 and 1 μg. PAS/ml. Survivors capable of growing on media containing more than 10 μg. PAS/ml. were not found among *c.* 10^9 viable cells of the parent strain tested.

Table 1. *Population structure of the parent strain not exposed to PAS. Test organism:* Mycobacterium tuberculosis *var.* hominis, *strain H 37 Rv*

Concentration of PAS (μg./ml.)	Number of survivors Dilution of cell suspension* 10^{-6}	10^{-4}	10^{-1}	10	Survival ratio
0	42·9 ± 6·04	M	M	M	1·00
0·01	36·0 ± 8·40	M	M	M	0·839
0·02	34·5 ± 6·15	M	M	M	0·840
0·05	32·2 ± 5·81	M	M	M	0·750
0·1	0·6	un	M	M	$1{\cdot}40 \times 10^{-2}$
0·2	0	un	0·5m	M	$1{\cdot}16 \times 10^{-7}$†
0·3	0	0	0·9	m‡	$2{\cdot}09 \times 10^{-7}$
0·5	0	0	0·3	3·3 ± 1·78	$7{\cdot}68 \times 10^{8}$
0·7	0	0	0	1·8 ± 0·47	$4{\cdot}19 \times 10^{-8}$
1	0	0	0	0·3	$6{\cdot}99 \times 10^{-9}$

* Number of survivors obtained by inoculating samples of cell suspensions to media containing different concentrations of PAS. (Mean of ten replicates) ± (standard deviation).

† Not accurate because of the membraneous growth appearing as a background.

‡ Distinguishable large colonies were not observed in these media. The phenomenon could be explained as an interference phenomenon of the growth of resistant colonies with that of many sensitive colonies which have appeared as a thin membraneous growth resulting from the retarded growth rate caused by PAS (Tsukamura *et al.* 1959*b*).

un, uncountable; m, thin membraneous growth; M, membraneous growth.

Population structures of clones derived from single colonies grown on media containing 0·3–1 *μg. PAS/ml.*

Clones were obtained by the first-step selection from the parent strain with 0·1–1 μg. PAS/ml. Two to five single colonies were picked from media containing 0·1, 0·3, 0·5, 0·7, and 1 μg./ml., and were subcultured on media without drug to form large populations. The resulting clones were designated P0·1R, P0·3R, P0·5R, P0·7R, and P1R, respectively. Population structures of these clones were analysed and some characteristic results are shown in Table 2.

Table 2. *Population structures of clones derived from single colonies selected from media containing different concentrations of PAS, which were inoculated with the parent strain*

PAS	Numbers of survivors from clones*				
(μg./ml.)	P0·1R	P0·3R	P0·5R	P0·7R	P1R
0	55·5 ± 10·4	137·6 ± 33·2	40·5 ± 3·54	32·8 ± 6·34	78·1 ± 21·8
0·3	0	115·8 ± 12·1	34·3 ± 7·73	31·5 ± 4·54	50·2 ± 15·9
0·5	0	110·0 ± 21·0	26·9 ± 5·69	30·8 ± 5·70	42·2 ± 7·42
0·7	0	121·7 ± 24·8	30·0 ± 4·98	34·6 ± 2·79	42·3 ± 12·9
1	0	104·7 ± 27·8	20·4 ± 7·32	35·6 ± 4·91	44·8 ± 8·33
2	0	45·2 ± 21·1	12·8 ± 4·16	24·8 ± 4·59	61·3 ± 14·2
3	0	2·2†	8·4 ± 2·16†	14·0 ± 3·94†	11·7 ± 9·41†
5	0	0	0	0	0
10	0	0	0	0	0

* (Mean of ten replicates) ± (standard deviation). Clones designated P0·1R, P0·3R, P0·5R P0·7R, P1R were picked from media containing 0·1, 0·3, 0·5, 0·7, and 1·0 μg./ml. PAS, respectively, after inoculating with parental strain. † Small colonies.

All the clones tested, excepting P0·1R and some of P0·3R (not shown in Table 2) showed similar population structures, although they had been obtained on media containing different concentrations of PAS. The degree of resistance of the clones was about 2 or 3 μg. PAS/ml. As mentioned previously, this suggests that these clones had the same genotype. The mutation frequency (appearance rate of resistant mutants among total viable population) to this phenotype from the wild phenotype appears to be about 10^{-7} to 10^{-8} (see Table 1).

The clone P0·1R represented a population structure distinctly different from that of the other clones, resembling that of the parent strain. This clone appears to have the wild phenotype, although an intermediate phenotype between that of the parental strain and of the clones P0·3R to P1R has not been excluded.

Two types of colonies grew on media containing 0·3 μg. PAS/ml. by the first-step selection: some large colonies and some small colonies. Two clones derived from large colonies and three clones derived from small colonies were tested for their population structures. The former showed a degree of PAS resistance of 2–3 μg. PAS/ml. and were considered to have the resistant genotype different from the wild genotype. On the other hand, the latter showed a degree of resistance up to 0·3 μg. PAS/ml. only. These clones were again subcultured on drug-free media and analysed for their population structures. On this occasion these clones showed a degree of resistance up to 0·1 μg. PAS/ml. It therefore appeared that the properties of the original small clones surviving on media containing up to 0·3 μg. PAS/ml. were only fluctuative and may not be due to a change of genotype.

Survivors capable of growing on media containing 10 and 100 μg. PAS/ml. were found in the clones P1R at rates of about 10^{-6}. An example of these experiments is shown in Table 3. Two additional experiments made independently showed that the appearance of survivors growing on media containing 10 μg. PAS/ml. among the total viable population were $1{\cdot}78 \times 10^{-6}$ and $5{\cdot}41 \times 10^{-6}$ and those of survivors growing on media containing 100 μg. PAS/ml. were $5{\cdot}22 \times 10^{-7}$ and $1{\cdot}29 \times 10^{-6}$.

Table 3. *Rate of appearance of mutants resistant to* 10 *and* 100 *μg. PAS/ml. in the clone P*1*R obtained by the first-step selection of the parent strain with* 1 *μg. PAS/ml.*

PAS (μg./ml.)	Number of survivors at a dilution of cell suspension*		Survival ratio
	10^{-5}	10	
0	47·8 ± 18·7	Membraneous	1·00
1	48·7 ± 18·2	Membraneous	1·02
10	0	10·0 ± 9·01	$2{\cdot}09 \times 10^{-6}$
100	0	3·4 ± 2·41	$7{\cdot}10 \times 10^{-7}$
1000	0	2·7 ± 1·79	$5{\cdot}65 \times 10^{-7}$
2000	0	0	$< 2{\cdot}09 \times 10^{-8}$

* (Mean of ten replicates) ± (standard deviation).

Table 4. *Population structures of clones, P*10*R and P*100*R, derived from single colonies of the clone P*1*R* (*see Table* 3) *grown on media containing* 10 *and* 100 *μg. PAS/ml.*

PAS (μg./ml.)	Number of survivors (survival ratio)	
	P10R	P100R
0	81·5 ± 15·7 (1·00)	19·4 ± 8·75 (1·00)
1	63·0 ± 4·79 (0·77)	15·5 ± 4·36 (0·80)
10	15·5 ± 5·16 (0·19)	6·6 ± 2·49 (0·34)
100	10·0 ± 1·76 (0·12)	4·7 ± 2·56 (0·24)
500	13·8 ± 5·45 (0·17)	5·0 ± 2·49 (0·26)
1000	1·7 ± 0·49 (0·02)	2·9 ± 2·29 (0·15)
2000	0	0

* (Mean of ten replicates) ± (standard deviation).

Population structures of clones derived from single colonies grown on media containing 10 *and* 100 *μg. PAS/ml.*

Single colonies were picked from media containing either 10 or 100 μg. PAS/ml., after inoculation from the clone P1R, and subcultured on drug-free media. The resultant clones were named P10R and P100R, respectively. These clones represented a population structure different from that of the original P1R, from which they were derived, but they were similar to each other. The results shown in Table 4 indicate that these clones, P10R and P100R, have a new phenotype producing a degree of resistance up to 1000 μg. PAS/ml. Two additional experiments made independently also gave similar results.

Population structure of a clone derived from a single colony grown on media containing 1000 *μg. PAS/ml.*

A single colony was picked from the surviving colonies of the clone P 100 R which had grown on media containing 1000 μg. PAS/ml. and subcultured on drug-free media to form a large population. The resulting clone, P 1000 R, was analysed for its population structure, which was found to be similar to clone P 100 R from which it was derived (Table 5). This means that clones P 100 R and P 1000 R have the same phenotype.

Table 5. *Population structure of clone, P* 1000 *R, derived from a single colony of the clone P* 100 *R (see Table* 4*) grown on media containing* 1000 *μg. PAS/ml.*

PAS (μg./ml.)	Number of survivors at dilution of cell suspension*		Survival ratio
	10^{-3}	10	
0	108·9 ± 12·7	Uncountable	1·00
1	91·6 ± 15·6		0·840
10	86·8 ± 19·2		0·796
100	60·0 ± 10·3		0·550
500	56·8 ± 13·2		0·522
1000	16·6 ± 10·0		0·152
2000	0	2·4 ± 1·95	$2{\cdot}20 \times 10^{-5}$
3000	0	0	$< 9{\cdot}18 \times 10^{-6}$

* (Mean of ten replicates) ± (standard deviation).

Table 6. *Population structure of a clone, P* 2000 *R, derived from a single colony of the clone P* 1000 *R (see Table* 5*) grown on media containing* 200 *μg. PAS/ml.*

PAS (μg./ml.)	Number of survivors*	Survival ratio
0	46·1 ± 10·1	1·000
1	50·3 ± 9·60	1·09
10	43·3 ± 8·62	0·940
100	43·5 ± 9·95	0·945
500	41·1 ± 8·83	0·892
1000	3·1 ± 2·56	$6{\cdot}74 \times 10^{-2}$
2000	0	$< 2{\cdot}17 \times 10^{-3}$

* (Mean of ten replicates) ± (standard deviation).

A few cells of the clone P 1000 R survived on media containing 2000 μg. PAS/ml. to produce colonies at a rate of about 10^{-5} (Table 5). In order to test whether these colonies surviving on media containing 2000 μg. PAS/ml. have a different phenotype from the mother clone, P 1000 R, the population structure of the clone P 2000 R derived from a single colony grown on media containing 2000 μg. PAS/ml. was analysed. If this clone had the same phenotype as the clone P 1000 R, that is, if it had survived on media containing 2000 μg. PAS/ml. by phenotypic variability, it should produce a population structure similar to that of the mother clone P 1000 R. However, if its phenotype were different to that of the mother clone, that is, if it had survived on such media by mutation, it should produce a different population structure from that of the mother clone. The results indicated that the population structure of the clone P 2000 R was similar to that of the clones P 10 R, P 100 R and P1000R (Table 6). This means that the clone P2000R has the same phenotype as the clones P10R, P100R and P1000R, in spite of the fact that the cell, from which the clone P2000 R was derived, had once grown on media containing 2000 μg. PAS/ml., and that its survival on media containing 2000 μg. PAS/ml. was produced by phenotypic variability.

Survivors on media containing 3000 μg. PAS/ml. were not found among about 10^5 viable cells of the clone P1000R (Table 5). It therefore appears that the limit of PAS resistance in the test organism may be 2000 μg./ml.

DISCUSSION

From the results obtained, it is suggested that three types of population structures exist showing three levels of clonal PAS resistance. The first is the population structure of the parent strain, the clone P0·1R, and some of the clones P0·3R, with a degree of resistance up to 0·2 PAS/ml. The second is the population structure of the clones P0·3R, P0·5R, P0·7R and P1R, with a degree of PAS resistance up to 3 μg. PAS/ml. The third is the population structure of the clones P10R, P100R, P1000R and P2000 P R, with a degree of PAS resistance up to 2000 μg. PAS/ml. It is evident that the second and the third types are produced by mutation, since survivors capable of producing these population structures appear discontinuously, and the property of PAS resistance is stably heritable. The second and the third population structures and degrees of resistance are thus considered to be produced by different genotypes from the wild genotype.

It is remarkable that clones derived from surviving colonies grown on different concentrations of PAS ranging from 0·3 to 2000 μg./ml. represented only two types of the population structures. This fact suggests that there are only two genotypes responsible for PAS resistance in the test organism.

Unlike the strains Aoyama-B *Mycobacterium tuberculosis* (Tsukamura, Noda, Yamamoto & Hayashi, 1959*a*, *b*), the genotype for higher resistance has not been found in the parent strain and has been found only in cells having the genotype for lower resistance in the test strain. The genotype for higher resistance may be produced by two mutations, the mutation of a gene responsible for lower resistance and the mutation of another gene that may act as a conditional factor.

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Morphological Variants of *Proteus hauseri*

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SUMMARY

Cultures of *Proteus hauseri* may consist of one or more of five colonial variants. Population pressure experiments started with one variant eventually yielded all other variants. Y variants form raised non-swarming colonies on a MacConkey-type agar at 37°, but swarm in concentric step-like rings at room temperature on this medium; they swarm in step-like concentric rings on nutrient agar at room temperature and at 37°. This variant is stable under conditions of logarithmic growth and is regarded as the wild type. Variant X is a typical Rough form and does not swarm under any circumstances. In continuous culture it mutates to the Y type at a high rate. The W variant forms colonies very similar to those of the Y variant, but like the X variant does not swarm. It is stable under conditions of continuous culture, but mutates on MacConkey agar to X and Y variants at low rates. On MacConkey agar at 37° the Z type appears as a flat amorphous colony surrounded by a continuous thin film of swarm. It also swarms at room temperature. This variant is replaced by a mixture of Y and W colonial forms in continuous culture experiments. Drop-like colonies, the 'Tröpfchenform' previously described by Weil & Felix (1917) were also encountered. Drop-like colonies are unpredictable in appearance and were not studied in detail.

Hanging-drop preparations of 4 hr. old broth cultures at 37° of either W or X colonial variants (which never swarm) reveal many motile forms. Young broth cultures of the other three variants consist of forms which are all actively motile. Lytic phage for a particular strain is active on all its variants except the X or Rough type which is also the only variant to form unstable suspension in saline and in broth. The Y, W and Z variants described here correspond reasonably well to the A, B and C phases of Belyavin (1951). The H, O terminology of Weil & Felix (1917) is not suitable and the nomenclature of Belyavin (1951) may be more satisfactory.

INTRODUCTION

Weil & Felix (1917) were the first to describe variants of the usual swarming type of Proteus. They designated the latter as H (Hauch) and the non-swarming slimy colonies as O (ohne Hauch). They also mentioned 'Tröpfchenform' or drop-like colonies. Other workers have described colonies of Proteus 'resembling those of *B. coli*' (Wenner & Rettger, 1919; Yacob, 1932) and Felix (1923), Moltke (1928) and Felix & Rhodes (1931) described what appear to be Rough colonial forms. Perch (quoted by Kauffmann, 1951) differentiated two types of colony on 0·1 % phenol agar: a 'dull' one which was flat and surrounded by a narrow swarming zone and a 'bright' one which was domed and sharply defined. Belyavin (1951) was the first to study morphological variants of the group systematically. Three colonial forms were described, which he provisionally labelled A, B and C phases. On nutrient agar overnight at 37° the A phase swarmed in a characteristic step-like concentric ring pattern. The B phase usually did not swarm while the C phase swarmed in a uniform film without step formation. Coetzee (1959*a*) described properties of a Rough form of a vulgaris variety of *Proteus hauseri* and mentioned other variants encountered. These were non-committally labelled with letters from the lower end of the alphabet. The present object is to characterize these variants and correlate the findings with those of Belyavin.

METHODS

The strains used were described by Coetzee (1958), and consisted of ten vulgaris and twelve mirabilis varieties of *Proteus hauseri*. The cultures were isolated during 1956 and have been maintained on nutrient agar slopes at 4°. The broth used consisted of 2 % (w/v) 'Lab-lemco', 4 % (w/v) Difco peptone and 0·5 % (w/v) sodium chloride at pH 7·4. The plating medium was a MacConkey-type agar made up as follows: Peptone (Oxoid) 40 g., bile salts (Oxoid) 3 g., sodium chloride 10 g., agar (B.B.L.) 34 g., distilled water 2000 ml. Dissolved in steamer and 20 g. of bacteriological lactose (Gurr), 6·0 ml. of 1 % (w/v) neutral red, and 2·0 ml. of 0·1 % (w/v) crystal violet added. pH adjusted to 7·2, and autoclaved at 15 lb. for 15 min. The method of initiation of cultures from single cells has been described (Coetzee, 1959*a*) and consisted of starting at least treble the number of cultures required with equal small inocula of 6 hr. broth cultures. If more than one-third of these cultures remain sterile it follows from the Poisson distribution that the remainder were initiated with an average of one viable organism. Continuous culture experiments consisted of maintaining logarithmic growth in cultures originally initiated with single organisms by subculturing *c*. 10^4 organisms at 8 hr. intervals into 800 ml. of fresh pre-warmed broth. All cultures were continually aerated and agitated by a stream of sterile air, and incubated at 37°. This method has also been described (Coetzee, 1959*a*). Population pressure experiments were done by starting a single organism culture in 40 ml. of broth. After 24 hr. incubation at 37°, 1 ml. of this culture (titre *c*. 4×10^8/ml.) was transferred to 40 ml. of fresh broth. This procedure allowed only 5–6 generations of growth before the M concentration was again reached. Further similar subcultures were done, and at intervals suitable dilutions were plated to determine the colonial morphology of the organisms present. The estimation of mutation rates by counting mutant outgrowths from parent colonies was as described for other systems (Ryan, Schwartz & Fried, 1955; Coetzee, 1959*b*, *c*; Coetzee & Sacks, 1960). Colonial morphology was studied by plating suitable dilutions of broth cultures on MacConkey-type agar plates (see Coetzee, 1959*a*). The saline stability of the organisms was tested by the method of Pandit & Wilson (1932) with concentrations of NaCl ranging from 0·4 to 5 %.

RESULTS

Morphological variants

All the strains examined were found to consist of one or more of five colonial variants. The variants showed no predilection for mirabilis or vulgaris strains.

Y variants. After 16 hr. incubation on MacConkey-type agar at 37° these colonies are 3–4 mm. in diameter, dome-shaped, circular in outline with an entire or slightly striated edge and possess a beaten copper surface. There is usually no swarming at 37° on this medium although sometimes, especially after longer periods of incubation, the edge may be less well defined and merges with *c.* 1 mm. of swarming spread. After 2 hr. at room temperature (18–24°) these colonies are surrounded by a halo of diffuse spread *c.* 2 cm. in diameter (Pl. 1, fig. 1). Swarming appears earliest between crowded colonies. After 24 hr. at room temperature the colony assumes the well-known step-like concentric ring appearance associated with Proteus (Pl. 1, fig. 2). Rauss (1936) demonstrated a very similar temperature effect on the swarming tendency of *Proteus morgani*. MacConkey plates inoculated with Y variants and kept at room temperature overnight show step-like concentric ring swarming. Swarming on blood agar or nutrient agar plates kept at 37° or room temperature for 16 hr. is always concentric and step-like. Increased concentrations of agar tend to diminish the diameter of the rings of swarming and diminishing the concentration has the reverse effect; below 1 % swarming is continuous.

Z variants. After 16 hr. incubation on MacConkey-type agar at 37° these colonies differ markedly from the Y type. They are usually larger and flatter and possess a finely granular surface. The outline is irregular and the edge merges with a thin film of swarming at least 2 cm. in diameter. If the colonies are now kept at room temperature they become amorphous and the uniform film of swarming spreads showing very little, if any, of the concentric step-like appearance of Y variants (Pl. 1, fig. 3). Swarming is more pronounced on blood agar and nutrient agar than on MacConkey agar. Concentrations of agar above 2 % diminish the swarming and make the latter more Y-like. Swarming of both Y and Z variants is usually more extensive at room temperature than at 37°. Y and Z variants of the same strain swarm into one another without a line of demarcation.

X variants. These are similar to the Rough colonial form of strain 15 previously described (Coetzee, 1959*a*). After 16 hr. incubation at 37° on MacConkey-type agar they are larger and flatter than a Y variant of the same strain. The outline is irregular and the edge is concave and bevelled. The surface is rough (Pl. 1, fig. 4). These colonies do not swarm under any circumstances.

W variants. These variants were referred to previously (Coetzee, 1959*a*) but not described. After 16 hr. on MacConkey-type agar at 37° they are smooth, shiny, with a circular outline, more heaped and slightly smaller than the corresponding Y colony (Pl. 1, fig. 5). They have a well-defined edge and like the X variety do not spread with any combination of temperature and medium tested. It is not always easy to distinguish overnight growths at 37° of Y and W colonies on MacConkey agar. The best way of doing this is to leave the plates at room temperature for a few hours when Y colonies develop their characteristic halo of spread (Pl. 1, fig. 1). After 2–5 days many W colonies on MacConkey agar at room temperature develop a variable number of outgrowths from their surfaces (Pl. 2, fig. 6). The outgrowths are of two kinds; one type resembles the step-like spread of a Y variant while the other has the rough surface associated with X variants. Subcultures of the former yield Y colonies and those of the latter, X colonies (see later).

Drop-like variants. After 16 hr. incubation on MacConkey-type agar at 37° these variants consist of a Y-like mother colony surrounded by numbers of small colonies (Pl. 2, fig. 7). Between constituent colonies of each galaxy there is usually a thin film of spread detectable. This assumes the typical Y swarming pattern after a further 18 hr. at room temperature (Pl. 2, fig. 8). These drop-like colonies only appear at 37°. Duplicate plates inoculated and left at room temperature show Y-like swarming without any droplet formation. The colonies do not breed true and usually become Y variants on subculture. As a result of their unpredictable and transient appearances they have not been extensively studied. These variants resemble the 'Tröpfchenform' colonies mentioned by Weil & Felix (1917). The small colony variants, hardly noticeable after 24 hr. growth, described by Felix (1923), were not encountered. No biochemical differences have been detected between morphological variants of any one strain.

Lytic phage isolated for each of the thirty-one strains (Coetzee, 1958) was active on all constituent variants of a strain, apart from the X variants which were always phage resistant. With the exception of X forms, colonies from all variants on nutrient agar formed stable suspensions in saline and in broth. The X form rapidly deposits at the bottom of the tube leaving the supernatent fluid clear.

Hanging-drop preparations of 4 hr. broth cultures at 37° reveal Y and drop-like variants as actively motile rods; the Z type consists mainly of motile filaments. The W form consists of cocco-bacillary and bacillary forms, *c.* 80 % of which are motile. X-types under similar conditions consist of bacillary forms only, *c.* 40 % of which are motile. After further periods of incubation the motility of all variants decreases both quantitatively and qualitatively. It has however been established that the W and X colonial variants, which do not swarm, certainly show many motile forms in young broth cultures.

To examine whether the five colonial variants described are peculiar to old stored cultures, a number of freshly isolated strains of *Proteus hauseri* were studied. These invariably consisted of one or more of the above-mentioned variants.

Interrelationships

Population pressure experiments. In these experiments, cultures initiated with a single organism of a particular morphological variant, in due course produced the whole gamut of morphological types. The variants did not appear in any set sequence or combination and it was impossible to determine the detailed lineage of a particular morphological variant from these qualitative experiments. Y and W colonial variants usually predominated in the complex conditions prevailing in the ageing and starving populations.

Continuous culture experiments. Quantitative results are only available for strain 15 (vulgaris) but have been qualitatively confirmed for mirabilis strains 34 and 13. Coetzee (1959*a*) described how a culture initiated from a single X-variant organism of strain 15 and maintained in logarithmic growth was gradually replaced by Y colonial variants; the process was complete after 350 hr. Experiments maintained for a further 500 hr. showed the Y type to be stable under the prevailing conditions.

The random occurrence of Y from X variants was established by means of fluctuation tests (Coetzee, 1959*a*), and the mutation rate involved was determined by means of continuous cultures. This proved to be $5{\cdot}3 \times 10^{-3}$/bacterium/generation. Continuous cultures started with single W variant organisms were also perfectly stable in experiments lasting for 300 hr. Similar experiments conducted with a Z colonial type resulted in its gradual replacement by a mixture of Y and W variants. The process was complete within a period ranging from 300 to 500 hr. for different Z strains. In different experiments (even with the same Z strain) the sequence with which the Y and W variants was detected varied and one variant might precede the other by as much as 48 hr. Frequently they were detected simultaneously. It would appear as if, under the particular conditions prevailing in continuous culture experiments, Z variants can independently give rise to Y and W colonial forms. A test to prove the mutational origin of these variants from the Z type has not been devised. The ratio of Y to W variants varied in different experiments but was maintained at a constant value for as long as has been investigated (200 hr.). This finding was confirmed for strain 15 by doing reconstruction experiments. Continuous culture experiments were started with a known ratio of the two variants. The initial ratio was maintained for long periods of time.

Table 1. *Results of an experiment to determine the mutational origin and mutation rates of X and Y variants from W variants of Proteus strain* 15

	Variant colonies (no.)	
	X	Y
No outgrowths	1759	1569
1 outgrowth	440	569
2 outgrowths	72	124
3 outgrowths	5	14
4 outgrowths	2	2
Mean no. outgrowths/colony (m)	0·226	0·381
Probability of drawing a similar sample of outgrowths from a Poisson distribution with m as mean (P)	0·22	0·34
Mutation rate/bacterium/generation ($m \ln 2/N$)	2×10^{-11}	$2{\cdot}9 \times 10^{-11}$

N = mean total bacterial count of five 5-day W colonies showing no outgrowths $= 9{\cdot}2 \times 10^{9}$.

Outgrowths of W colonies. Counts of the number of Y and X outgrowths from W colonies of strain 15 after 5 days at room temperature are presented in Table 1. After 5 days at room temperature no more outgrowths appeared. One hundred and ten W colonies which showed no outgrowths at this stage were cut out and each *entire* colony spread on the surface of a separate MacConkey agar plate. After 16 hr. at 37° and a further 8 hr. at room temperature plates were examined for swarming growth. Only three plates showed swarming. This implies that the mean number of undetected Y outgrowths per colony is negligible. A simple test for concealed Z growths was not devised. The P values given in Table 1 represent the chance of drawing a similar sample from a Poisson distribution with a mean equal to the observed mean number of outgrowths per colony. The values obtained are taken to indicate that the outgrowths were randomly distributed and support the theory of the mutational origin of events which started the clonal outgrowths (Ryan *et al.* 1955). Mutation rates are also presented in Table 1; these are low and may be over-estimated because acid-Giesma staining (Bisset, 1950) of W organisms reveals 1–4 chromatinic bodies per organism. These low mutation rates are compatible with the fact that under conditions prevailing in continuous culture, W organisms are stable.

DISCUSSION

The microscopic pleomorphism which induced Hauser (1885) to name these organisms after the Homeric sea deity Proteus is reflected in variations of colonial morphology. It becomes a matter of some interest to determine which is the wild type. It has been established that on solid media Y and X variants arise from the W form. The mutational origin of these two variants from the W form has been established for strain 15 and the mutation rate determined. The mutation rate of the X to Y form has also been determined for strain 15 (Coetzee, 1959*a*). In continuous cultures the Z form is replaced by a mixture of Y and W variants. The latter combination is also most prevalent in population pressure experiments. Many of these experiments have been conducted with variants of only one strain, but there is no reason to doubt the general applicability of results obtained. These results suggest that the Y variant is the modal type. The frequency with which this variant is isolated from clinical material supports this suggestion. The H form of Weil & Felix (1917) corresponds either to our Y or Z variants. The descriptions of their O form and of the coliform-like colonies of Wenner & Rettger (1919) and Yacob (1932) may refer to our W variant, but it is difficult to relate the variants described by Perch (Kauffmann, 1951) to the present varieties.

Belyavin (1951) did not mention the effect of temperature on the swarming phenomenon of his A phase on MacConkey agar, yet the characteristics of A phase swarming on nutrient agar appear to be identical with those of the Y variants described here. Other features agree and Belyavin's phase A must be considered very similar to our Y variants. Belyavin regarded this phase A as the modal form. The W variants described here resemble Belyavin's B phase. Differences are that some of his young cultures spread on nutrient agar and some were unstable in saline. He regarded the B phase as showing some Rough characteristics. We have not been able to confirm this since our W variants are stable in saline and broth, and are susceptible to phage active on the corresponding wild type. We have not, however, done any serological tests. Belyavin's C phase did not swarm on MacConkey agar. This may have been due to differences in ingredients of media used. Some of his C-phase colonies were unstable in saline. However, the type of swarming on nutrient agar was like that of our Z variants. The microscopic morphology was also identical and the C phase and the Z variants can be regarded as similar. If the foregoing is accepted then we have demonstrated, by means of continuous cultures, a direct transition from the C to the B phase, a change which Belyavin did not report.

Despite Krikler's (1953) demonstration of the unreliability of employing the C (Z) phase, the fact that A (Y) and C (Z) variants of a particular strain swarm into one another without a line of demarcation may be taken as support (Dienes, 1946) of Belyavin's finding of antigenic similarity between his A and C phases.

On the question of nomenclature, the O and H terminology of Weil & Felix (1917) deserves precedence. By common usage the OH → O variation implies loss of flagellar antigens. Reference to our W or to Belyavin's B forms, which show many motile organisms, as O forms would therefore be misleading. Weil & Felix did not distinguish between the two varieties of swarmers described here and their H term is thus ambiguous. The X colonial type is almost certainly an R variant in the terminology of Arkwright (1920, 1921) and, although no serological studies have been done, the latter symbol should be applied. Close identity has been demonstrated between our Y, W and Z variants and Belyavin's A, B and C phases, respectively. While many of Belyavin's strains had been maintained in stock culture for over 10 years, our cultures were less than 3 years old when examined and have never been dried. Moreover, newly isolated strains showed the same phenomena. It is possible that differences encountered may be explained on the basis of age of cultures examined; if this should prove to be so then it is recommended that Belyavin's terminology be adopted.

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EXPLANATION OF PLATES

Plate 1

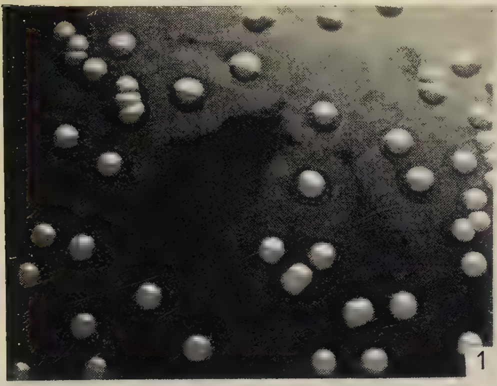
Colonial morphology of *Proteus hauseri* variants on MacConkey-type agar. All figures life size.

Fig. 1. Y variants after 16 hr. incubation at 37° followed by 2 hr. at room temperature.
Fig. 2. Y variants after 24 hr. at room temperature.
Fig. 3. Z variants after 24 hr. at room temperature.
Fig. 4. Four X variants and two W variants, after 16 hr. at 37°.
Fig. 5. W variants after 16 hr. at 37°.

Plate 2

Colonial morphology of *Proteus hauseri* variants on MacConkey-type agar.

Fig. 6. W variants. 5-day colony showing Y-type (left) and X-type (right) outgrowths; magnification *c.* × 6.
Fig. 7. Drop-like variants after 16 hr. at 37°; magnification *c.* × 1·5.
Fig. 8. Drop-like variants after 16 hr. at 37° followed by 18 hr. at room temperature. Life size.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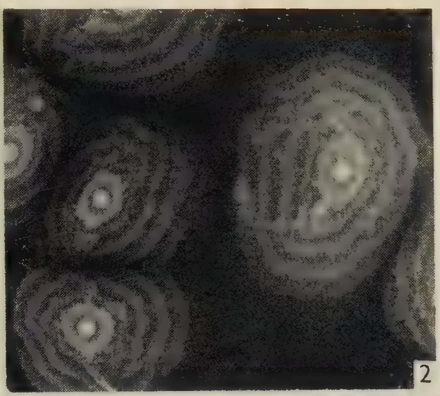
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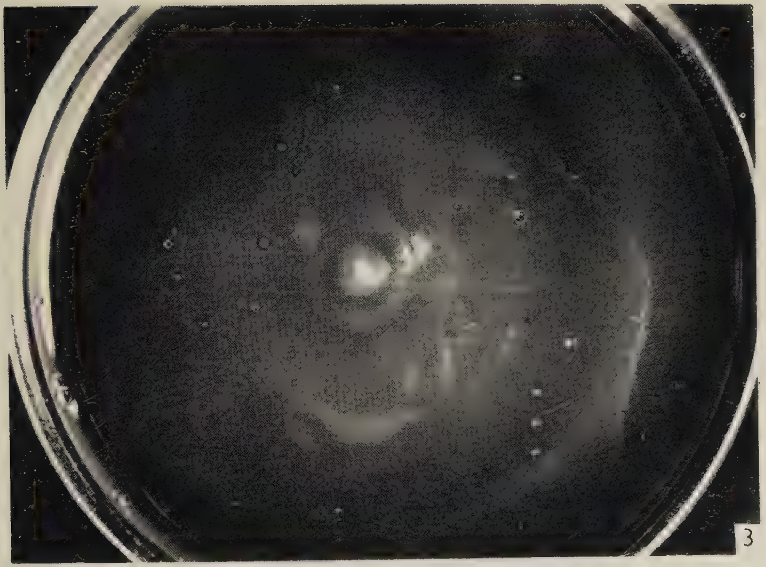
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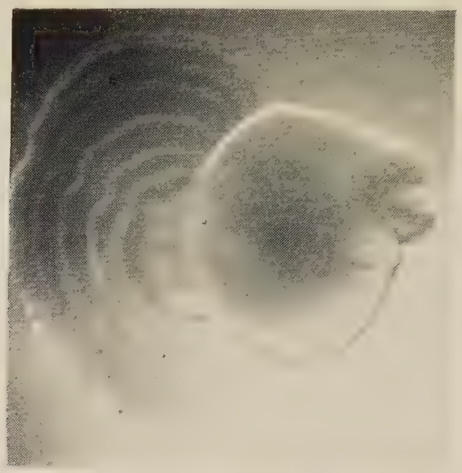

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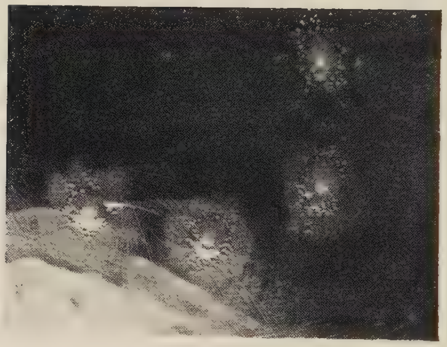

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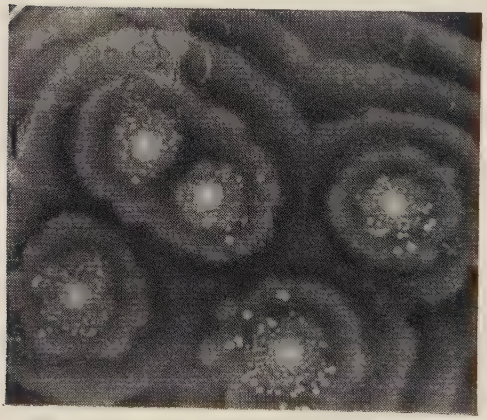

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Nutrition of *Flavobacterium aquatile* Strain Taylor and a Microbiological Assay for Thiamine

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SUMMARY

Flavobacterium aquatile strain Taylor grew readily in a medium containing (%, w/v); trypticase, 0·5; glucose, 0·5; K_2HPO_4, 0·05; KH_2PO_4, 0·05; $MgSO_4.7H_2O$, 0·02; supplemental thiamine. Growth was proportional to the thiamine-HCl content in the range 1–80 mμg./10 ml. medium. Larger quantities of thiamine did not increase growth. Maximum growth responses at all concentrations of thiamine were obtained after 48 hr. incubation at 30° when the cultures were shaken during incubation. The response of *F. aquatile* to thiamine gives a basis for a microbiological assay. Of the compounds tested only thiamine pyrophosphate and acetylthiamine replaced thiamine. None of the other vitamins, or various purines, pyrimidines, nucleosides, or miscellaneous compounds tested except certain carbohydrates, altered the response to thiamine. Maltose or ribose (0·5 %, w/v) stimulated *F. aquatile* in the range 0–5 mμg. thiamine/10 ml. At greater concentrations of thiamine, and in the presence of glucose, slight growth inhibitions were caused by maltose and ribose. Sodium chloride at concentrations greater than 0·05 M retarded growth generally and therefore the response to thiamine was modified. None of the interfering compounds appeared to invalidate the use of the bacterium as an assay organism for thiamine.

INTRODUCTION

The measurement of thiamine in natural materials may be accomplished by chemical or biological methods, the latter being especially useful where small amounts of the vitamin are concerned. Techniques which have been proposed were reviewed by Mickelsen & Yamamoto (1958). In addition Deibel, Evans & Niven (1957) described a procedure using *Lactobacillus viridescens*; Maciasr (1957) reported an improved assay medium for use with *L. fermenti*; and Littman (1958) showed that the pathogen *Cryptococcus neoformans* could be used for thiamine assay. Of the several micro-organisms proposed, *L. fermenti* (Sarett & Cheldelin, 1944), *Kloeckera brevis* (Hoff-Jorgensen & Hansen, 1955) and *L. viridescens* (Deibel *et al.* 1957) seem best suited for routine assay. With the exception of mutant strains of *Escherichia coli* (Mickelsen & Yamamoto, 1958) the use of Gram-negative bacteria for such an assay has not been common. Investigations of the nutritional requirements of *Flavobacterium aquatile* strain Taylor (Lauro, 1955) showed a requirement for thiamine in a relatively simple basal medium. That study has been continued and a microbiological assay for thiamine dependent upon the growth of *F. aquatile* is presented here.

METHODS

Organism. Flavobacterium aquatile strain Taylor was originally obtained from Dr E. Windle Taylor (London) who made the isolation from the source described by Frankland & Frankland (1889) for *Bacillus aquatilis.* The strain has been studied (Weeks, 1955) but a detailed report of its nutritional requirements has not been made. The bacterium has been placed in several culture collections: the National collection of Type Cultures, London; the Northern Regional Research Laboratories, U.S. Department of Agriculture, Peoria, Illinois; the American Type Culture Collection, Washington, D.C.

Media. Stock cultures were maintained upon slopes of medium M-5 agar containing (%, w/v): Difco sodium caseinate, 0·2; Difco yeast extract, 0·05; Difco proteose peptone, 0·05; K_2HPO_4, 0·05; agar, 1·5; adjusted to pH 7·4–7·6. All media were prepared with twice-distilled water. The studies of the growth response to thiamine were made in tubes containing 10 ml. liquid medium (A-1) of the following (final) composition (%, w/v): K_2HPO_4, 0·05; KH_2PO_4, 0·05; $MgSO_4.7H_2O$, 0·02; glucose, 0·5; trypticase (a tryptic digest of casein; Baltimore Biological Laboratories), 0·5. A solution of the inorganic salts was prepared separately, adjusted to pH 7·4, and autoclaved (121°, 15 min.). The volume of water used in preparing the salt solution was between 50 and 90 % (v/v) of the ultimate volume, depending on the experiment. The glucose and trypticase were added aseptically to the salt solution in 0·5 ml. amounts from separately sterilized 10 % (w/v) stock solutions. Sterile solutions of thiamine and the various organic supplements being considered were then added, and finally the volume brought up to 10 ml. per tube with sterile water. After the composition of the assay medium was established, aseptic assembling of the medium was given up for conventional preparation and autoclave sterilization of the complete medium, except for thiamine or the samples being assayed. These latter were always added aseptically from sterile solutions. Usually bromthymol blue indicator was included in medium A-1. When any of the aseptic supplementations changed the pH value as shown by the indicator, the reaction was readjusted with sterile 2 % (w/v) HCl or NaOH solution.

Preparation of inoculum. Stock cultures of *Flavobacterium aquatile* growing upon M-5 agar slopes were subcultured every two weeks. Following inoculation, the cultures were incubated at 28–30° for 48 hr. and then stored at 10°. Cultures to be used for studies of growth response to thiamine were also grown upon M-5 agar medium. A 24 hr. stock culture was used to inoculate a sufficient number of slopes for an experiment. After 24 hr. incubation, the growth was suspended in sterile distilled water, adjusted to a standardized turbidity (optical density = *c.* 0·25, light path = 1 cm., λ = 550 mμ), and 0·1 ml. used to inoculate each tube.

Culture conditions. All studies of the nutritional requirements of *Flavobacterium aquatile* were done in test tubes (19 × 150 mm.) covered with Morton stainless-steel culture tube closures (Bellco Glass, Inc., Vineland, N.J.). The tubes were incubated at 28–30° on a rotary shaker adjusted to give 160 rev./min. Incubation times were 24–96 hr., depending upon the experiment.

Measurement of growth. The relative extent of bacterial growth was estimated from turbidity read as optical density on a Beckman Model B spectrophotometer set at a wavelength of 550 mμ. When the medium included bromthymol blue indicator, all tubes were acidified (0·1 ml. of N-HCl) before measurement of optical density. The cultures were poured into 1 cm. cuvettes (Corex) and the optical measurements were made by using the uninoculated medium as the reference.

RESULTS

Inorganic salts solution

Investigations of the response of *Flavobacterium aquatile* to thiamine were begun with the inorganic salt solutions A and C of Snell & Wright (1941). This mixture was supplemented with stock solutions of glucose and trypticase to 0·5 % (w/v) of each and with 10 μg. thiamine.HCl/tube. The complete medium contained 0·5 ml. each of stock solutions A and C per 100 ml. The inorganic compounds of the salt solutions (Table 1) were tested in several mixtures to determine the minimal mixture necessary. The results (Table 1; Fig. 1) showed that a mixture of the potassium phosphates and magnesium sulphate or the complete A and C mixture was required for maximum growth. The mixture of potassium phosphates and magnesium sulphate was chosen for subsequent experiments. The apparent inhibition by manganese and iron salts was not studied further.

Table 1. *Effect of inorganic salts upon the growth of* Flavobacterium aquatile

Inorganic constituents*	Growth after supplementation with organic compounds† (optical density)
A+C	2·09
A	1·64
C	1·36
A+$MgSO_4.7H_2O$	2·00
A+NaCl	1·66
A+$MnSO_4.H_2O$	0·64
A+$FeSO_4.7H_2O$	0·97
None	1·40

* A contained (mg./10 ml. medium) K_2HPO_4, 5·0; KH_2PO_4, 5·0. C contained (mg./10 ml. medium) $MgSO_4.7H_2O$, 4·0; NaCl, 0·2; $MnSO_4.H_2O$, 0·8; $FeSO_4.7H_2O$, 0·02. Single inorganic salts were used in same concentration in which they were found in C.

† Organic supplements (amt./10 ml. medium) were: trypticase, 50 mg.; glucose, 50 mg.; thiamine.HCl, 10 μg. Cultures were incubated at 30° for 48 hr. with continuous shaking.

Nitrogen source

Preliminary studies showed that neither various amino acid mixtures, nor acid-hydrolysed casein served as the nitrogen source. Various enzymic hydrolysates of casein were satisfactory; trypticase was chosen. Addition of glucose greatly increased the growth (Fig. 5). Within the first 48 hr. of incubation the optimal concentration of trypticase was 0·5 % (w/v). Trypticase at 1 % (w/v) markedly delayed growth during the initial 48 hr. of incubation, but this retardation disappeared by 96 hr. (Fig. 1). The reason for this retardation of growth at the higher peptone concentrations is not known, but it was found that after dialysis of the trypticase, the non-diffusible fraction did not show growth retardation at concentrations equivalent to 1 % trypticase (Fig. 1*c*). Flame photometric analysis (Perkins Elmer flame photometer) showed trypticase to contain sodium equivalent to 0·70 % NaCl and potassium equivalent to 0·36 % KCl; total ash was 6–7 %.

Response of Flavobacterium aquatile *to thiamine*

The growth response of *Flavobacterium aquatile* to thiamine in medium A-1 is shown in Fig. 2, upper curve. Thiamine was added aseptically to each tube as a sterile dilution prepared from a thiamine.HCl stock solution (10 mg./100 ml.). The stock solution was autoclaved for 10 min. at 121°. It was unnecessary to acidify the solution to prevent thiamine destruction during sterilization. Amounts of thiamine.HCl from 0·1 to 10,000 mμg./10 ml. medium were tested. Quantitative responses to thiamine.HCl occurred in the range from 1 to 50 mμg./10 ml. Maximum growth occurred when 80 mμg. of the vitamin were present, and increasing the concentration to 10,000 mμg. did not further increase growth. Between 50 and 80 mμg./10 ml. the rate of growth progressively decreased and the accuracy of the assay decreased. Although growth responses were apparent in the range of 0·1–1·0 mμg./10 ml., the accuracy of the assay may be questioned at these lower concentrations. The shape of the thiamine response curve has remained unchanged

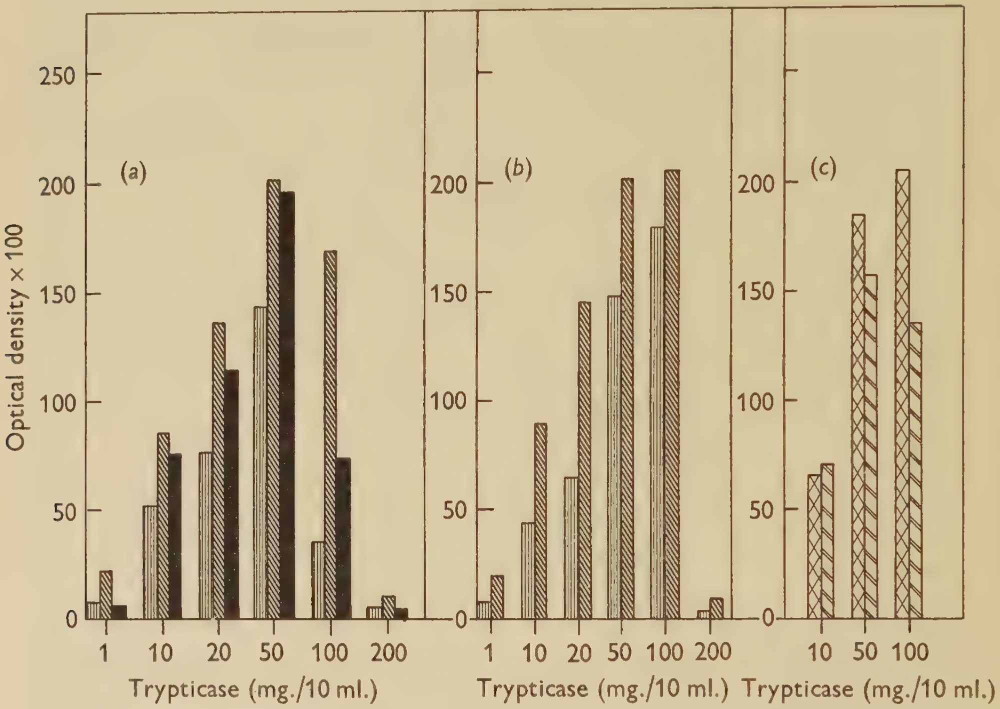

Fig. 1. Effect of trypticase content and inorganic basal medium composition on the growth of *Flavobacterium aquatile*. All media contained 0·5 % (w/v) glucose and 10 μg. thiamine/10 ml. (*a*) 48 hr. incubation; (*b*) 96 hr. incubation. ▥, no basal A, C, or Mg^{++}; ▧, basal A + Mg^{++}, ■, basal A + C. (*c*) 48 hr. incubation; basal A + C. ☒, non-diffusible trypticase fraction; ▨, diffusate trypticase fraction.

during a year or more of study. The maximum growth response to thiamine was obtained within 48 hr.; the optical density was unchanged by further incubation. For thiamine.HCl concentrations greater than 10 mμg./ml., maximum growth did not occur within the initial 24 hr. of incubation (Table 2).

Table 2. *Influence of incubation time upon the growth response of* Flavobacterium aquatile *to thiamine in medium A*-1

	Incubation time			
Thiamine HCl (mμg./10 ml.)	24 hr.	48 hr.	72 hr.	96 hr.
	Growth at 30° (optical density ×100)			
100	46	192	—	198
50	52	167	146	159
30	—	152	149	159
10	29	56	51	54
6	43	35	36	40
4	27	27	28	36
1	17	16	16	16
0·6	10	11	13	13
0·4	10	11	14	13
0·1	10	13	12	11
0·0	9	9	9	—

Influence of cultural history on the response of Flavobacterium aquatile *to thiamine*

The growth response of *Flavobacterium aquatile* to thiamine was very stable. Shortly after the organism was isolated in 1952, a number of cultures were lyophilized. In addition, the parent culture and selections from platings of it were maintained as growing cultures. During a 5-year period, 9 separate clones have been maintained and all have responded similarly to thiamine. A lyophilized culture was activated, subcultured twice and then tested for its growth response to thiamine; the response was typical. One of the cultures had been given to a colleague who maintained it by infrequent subculture; this clone was tested and also gave a growth response to thiamine which was indistinguishable from that shown in Fig. 2, upper curve.

Inocula for the thiamine growth response studies were usually distilled water suspensions from M-5 agar slope cultures and were used without washing. In one group of experiments the inocula were washed with phosphate buffer, but the results were not different from those shown in Fig. 2, upper curve. The trace of growth in medium A-1 without added thiamine.HCl, was not decreased. When stock cultures were subcultured monthly and the growth from 24 hr. subcultures used to inoculate medium A-1, the maximum growth response was equivalent to optical density *c.* 1·7; with weekly or more frequent subcultures, the maximum growth increased to optical density *c.* 2·3. With the increase in optical density the slope of the growth response curve increased.

The effect of sterilization of the assay medium on response of Flavobacterium aquatile *to thiamine*

Medium A-1 was usually prepared aseptically by mixing sterile stock solutions. Investigations were made of the growth responses of *Flavobacterium aquatile* to thiamine in the medium prepared in a variety of ways with respect to sterilization, to determine whether aseptic compounding was necessary. Results are shown in Fig. 2. A usable assay curve was obtained when the complete medium, except for thiamine, was autoclaved. It is evident that growth responses to thiamine were decreased when the assay medium was prepared in any manner other than assembly from separately sterilized stock solutions. The extreme decrease in growth response occurred when the vitamin was autoclaved with the medium and was doubtless due to partial destruction of the vitamin during sterilization.

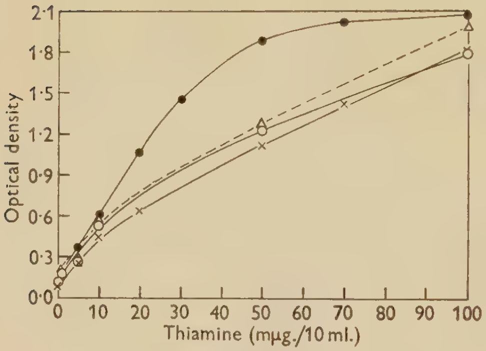

Fig. 2

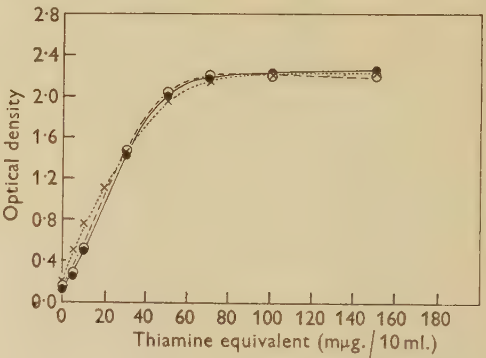

Fig. 3

Fig. 2. Effect on growth responses of *Flavobacterium aquatile* produced by autoclaving various mixtures of constituents of medium A-1. ●—●, basal medium autoclaved; trypticase, glucose and thiamine added aseptically. △--△, basal medium + trypticase autoclaved together; glucose and thiamine added aseptically. ○—○, basal medium + trypticase + glucose autoclaved together; thiamine added aseptically. ×—×, basal medium + trypticase + glucose + thiamine autoclaved together.

Fig. 3. Growth responses of *Flavobacterium aquatile* to various forms of thiamine in medium A-1. ×----×, thiamine pyrophosphate; ⊙—⊙, acetylthiamine; ●—●, thiamine.

Studies suggested that some of the decreased response to thiamine in the autoclaved medium was associated with reactions involving trypticase rather than glucose. A general decrease in the growth response to thiamine occurred irrespective of whether or not glucose was included in the medium before autoclaving (Fig. 2). In a different experiment medium A-1 was assembled from the previously sterilized stock solutions but the trypticase stock solutions had been heated for 2 hr. at 121°. The growth-response curve to thiamine resembled that which occurred when the inorganic salts solution and trypticase were autoclaved together. The decreases in growth response to thiamine were greater in the range 5–70 mμg./10 ml. than for lesser or greater amounts of thiamine. The consequence of this was an almost 50 % increase in the range of thiamine concentrations which could be measured from growth response.

The specificity of the requirement for thiamine

Thiamine pyrophosphate or acetylthiamine replaced thiamine quantitatively in medium A-1 (Fig. 3). The two moieties of thiamine, namely 2-methyl-4-amino-5-aminomethyl pyrimidine.2HCl and 4-methyl-5-β-hydroxyethyl thiazole, singly or together, were unable to replace thiamine (Table 3).

The remaining organic compounds tested did not replace thiamine or affect growth in the presence of thiamine. The compounds tested and the concentrations used in medium A-1 are listed following. Purines and pyrimidines (100 μg./10 ml.): guanine, adenine, xanthine, hypoxanthine, 2:6-diaminopurine, cytosine, thymine, uracil, orotic acid, and the nucleoside thymidine. Vitamins of the B-complex (filtered sterile): folic acid, biotin, vitamin B_{12}, thioctic acid (1 or 10 mμg./10 ml.); riboflavin-5-phosphate, riboflavin, calcium pantothenate, pyridoxal.HCl, pyridoxal phosphate, pyridoxamine.2HCl, pyridoxine.HCl, nicotinamide, nicotinic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, choline chloride (10 mμg. or 10 μg./10 ml.); inositol (100 or 500 μg./10 ml.). Mixtures of all of the purines and pyrimidines or all of the B-vitamins did not allow growth in the presence of thiamine or in its absence. Miscellaneous compounds: ureidosuccinic acid, kinetin, ascorbic acid, and sodium thioglycollate did not influence the response of the bacterium to thiamine.

Table 3. *Growth of* Flavobacterium aquatile *in media containing thiamine or its moieties*

Compound	Concentrations used (mμg./10 ml.)						
	100,000	100	50	10	5	1	0
	Growth (optical density × 100)						
Thiamine.HCl	224	220	201	53	33	20	12
2-Methyl-4-amino-5-aminomethyl pyrimidine.2HCl (A)	11	11	11	11	11	10	9
4-Methyl-5-β-hydroxyethyl-thiazole (B)	9	9	9	10	12	9	10
(A)+(B)	9	9	9	—	10	10	9

Carbohydrates. The carbohydrates shown in Table 4 were tested for their effects upon the response of *Flavobacterium aquatile* to thiamine. The results show that fructose, maltose and the pentoses when added to the glucose + thiamine-containing medium increased bacterial growth beyond that of the glucose control. Similar effects were evident in the absence of glucose, except for arabinose. Ribose and maltose supported some growth in the absence of thiamine and this was studied more extensively.

The ribose and maltose effects were studied by adding 0·5 % (w/v) ribose or maltose to medium A-1, already containing 0·5 % glucose, or by substituting ribose or maltose for the glucose. Influence of carbohydrate concentration *per se* was determined from growth responses in medium A-1 containing either no added carbohydrate, or 0·5 or 1·0 % glucose. The results are shown in Figs. 4, 5. The mixtures of ribose + glucose or maltose + glucose increased growth beyond that of the

Table 4. *Influence of carbohydrates upon the response of* Flavobacterium aquatile *to thiamine in medium A*-1

Carbohydrate*	Thiamine present† Glucose	Thiamine present† No glucose	No thiamine or glucose
	Growth response (optical density × 100)		
Xylose	47	32	10
Arabinose	44	13	8
Ribose	124	94	62
Fructose	58	24	9
Galactose	37	15	7
Maltose	168	52	23
Sucrose	27	9	8
Lactose	37	18	8
None	40	18	8

* Final concentration = 0·5 % (w/v).

† Thiamine concentration = 6 mμg. /10 ml.

control when concentrations of thiamine.HCl were less than 10 mμg./10 ml. (Fig. 4). With greater amounts of thiamine the growth was less than that of the glucose control. The slight stimulatory effects of ribose and maltose were apparent whether or not thiamine was added (Figs. 4, 5). The possibility that the ribose and maltose used were contaminated by minute amounts of thiamine was not excluded. Ribose seemed unavailable as an energy source but maltose was satisfactory. The mixtures glucose + maltose or glucose + ribose decreased the growth response to

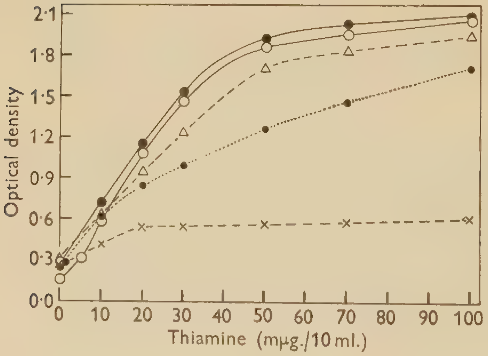

Fig. 4

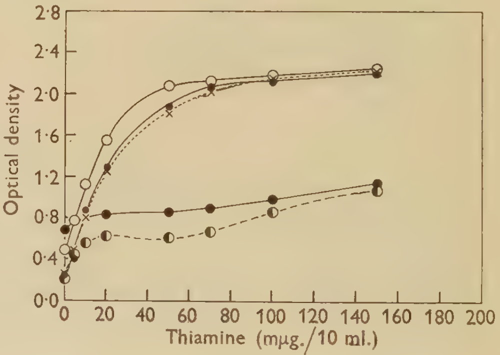

Fig. 5

Fig. 4. Effect of various carbohydrates on the growth response of *Flavobacterium aquatile* to thiamine. Systems contained basal medium A + Mg^{++}, 0·5 % (w/v) trypticase, and 0·5 % (w/v) of each carbohydrate as follows: ●—●, maltose; ○—○, glucose; △—△, glucose + ribose; ●----●, glucose + maltose; ×—×, ribose. All supplements added aseptically from sterile stock solutions.

Fig. 5. Effect of various carbohydrates on the growth response of *Flavobacterium aquatile* to thiamine. All supplements added aseptically to basal medium A + Mg^{++} from sterile stock solutions. Systems contained 0·5 % (w/v) trypticase plus the following % (w/v) carbohydrates: ◐—◐, no added carbohydrate; ●—●, 0·5 % ribose; ●—●, 0·5 % glucose; ×----×, 1·0 % glucose; ○—○, 0·5 % maltose.

thiamine when compared with that which occurred in the medium containing only glucose. The reason for this is not known.

The principal problem, however, was to define the degree to which ribose and maltose would interfere with microbiological assays for thiamine when using medium A-1. Usually this medium was supplemented with different amounts of thiamine which were prepared from a sterile stock solution using water as the diluent. To study the effects of maltose and ribose as if these compounds were present in natural substances subjected to assay, the first 10-fold dilution of the stock thiamine.HCl (10 mg./100 ml.) was made in 1 % (w/v) maltose or ribose solution; the subsequent dilutions were made with sterile water. In this manner, amounts of either carbohydrate (1–100 mg.) were added to each tube along with the thiamine; the results are shown in Fig. 6. It would seem that with the experimental conditions used, neither maltose nor ribose influenced the accuracy of the assay.

Influence of NaCl. Flavobacterium aquatile appeared to be unusually sensitive to electrolytes; its sensitivity to NaCl is shown in Fig. 7. Under conditions allowing maximum growth (0·5 %, w/v, trypticase) addition of 0·2 M-NaCl essentially stopped growth; perceptible inhibition occurred in media containing 0·01 M-NaCl. This inhibitory effect was more pronounced with smaller concentrations of trypticase. Sodium chloride was not a specific inhibitor; similar results in different degrees were obtained with Na_2SO_4, KCl, K_2SO_4, $MnCl_2$, $MgSO_4$, $CaCl_2$, Na acetate, and Na citrate. The effect is not explainable in terms of solute pressure since amounts of sucrose with much greater solute pressures did not inhibit growth.

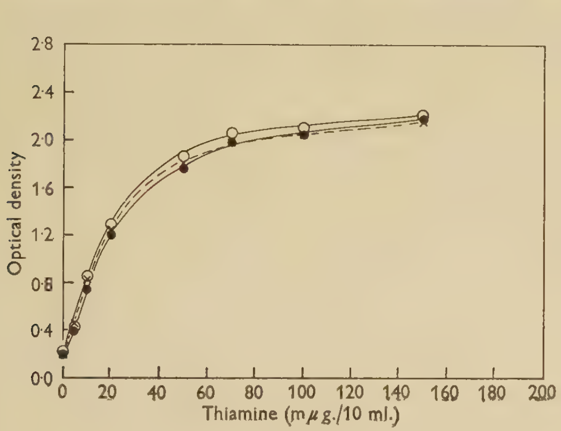

Fig. 6

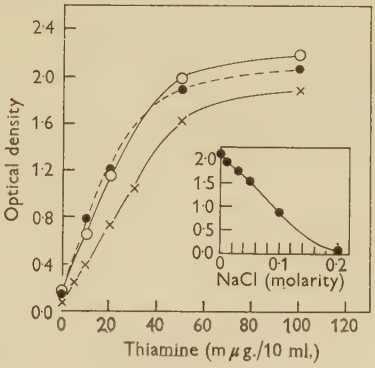

Fig. 7

Fig. 6. Effect of constant ribose:thiamine and maltose:thiamine ratios on the growth response of *Flavobacterium aquatile*. Stock thiamine solution contained 1·0 mg. thiamine/100 ml. of 1 % ribose or maltose. Subsequent dilutions made with distilled water. All systems contained basal medium $A+Mg^{++}$, 0·5 % trypticase, and 0·5 % glucose (w/v). ○—○, control (all thiamine dilutions in water); ×---×, maltose added with thiamine; ●—●, ribose added with thiamine.

Fig. 7. Growth response curves of *Flavobacterium aquatile* to thiamine in presence of different NaCl concentrations. Systems contained basal medium $A+Mg^{++}$, 0·5 % trypticase, 0·5 % glucose (w/v), and the following molar concentrations of NaCl: ○—○, control (no NaCl); ●---●, 0·02 M-NaCl; ×—×, 0·05 M-NaCl. Fig. 7 (inset) shows growth response of *F. aquatile* to thiamine in above control medium containing the indicated molar concentrations of NaCl.

An attempt was made to establish the degree to which NaCl might interfere with the microbiological assay. The usual experimental conditions were used to obtain a standard thiamine-response curve. Two additional experiments were arranged in which 0·02 M or 0·05 M-NaCl was included in medium A-1. The results (Fig. 7) show some inhibition by NaCl. The curves also show the typical thiamine responses. It would be possible to correct for electrolyte interference in microbiological assays by using a proper reference curve but it seems probable that dilution of the samples to be assayed would make the electrolyte effect unimportant.

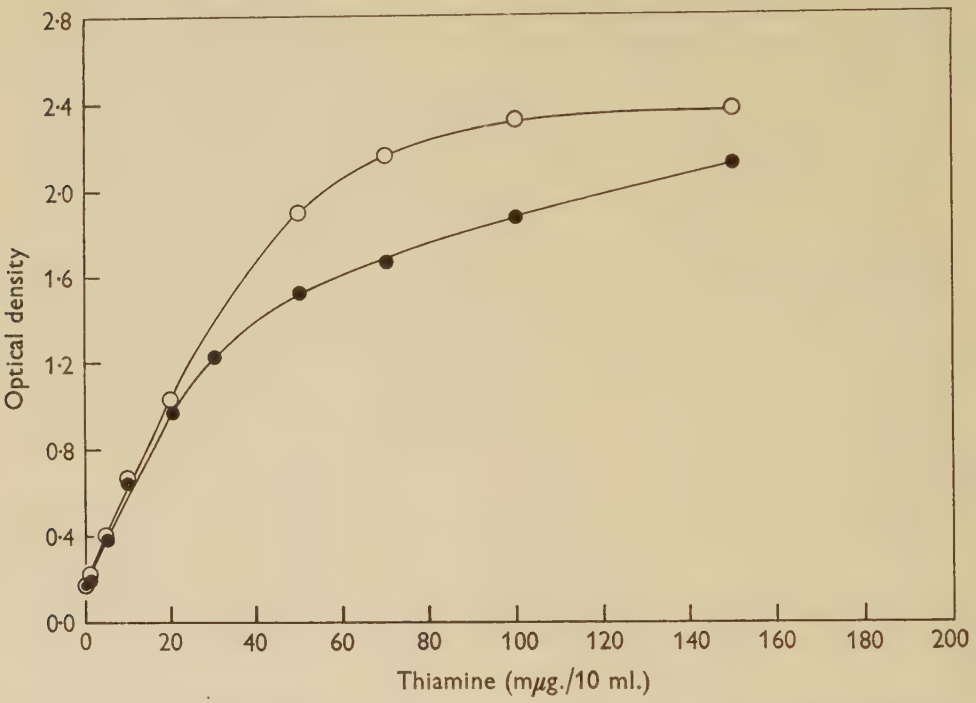

Fig. 8. Standard curve for thiamine recovery experiment using *Flavobacterium aquatile* as assay organism. ●—●, actual assay curve with all constituents of medium A-1 except thiamine sterilized together. ○—○, comparison curve showing response of *F. aquatile* to thiamine in medium A-1 compounded aseptically from sterile constituents.

Microbiological assay for thiamine and thiamine recovery experiments

Thiamine recovery experiments were arranged and the procedure given below could be used for routine measurements of thiamine. The assay medium was medium A-1; the inorganic salts, trypticase and glucose solutions were mixed; the volume brought to 90 % (v/v) of that ultimately sought, the pH adjusted to 7·4, and the medium tubed in 9 ml. amounts. Sterilization was by autoclaving at 121° for 15 min. The samples to be assayed were prepared as water extracts when the substance being tested was dry. Appropriate stock dilutions, adjusted to pH 7·0, were prepared for each material and sterilized by autoclaving. Further dilutions were prepared aseptically from the sterile stock solutions and 1 ml. of each sample dilution added to the tube assay medium. The bacterial cultures used for the inoculum were 24 hr. cultures which originated from similar 24 hr. cultures which had been inoculated from 7-day parent cultures held at 15°. All these cultures for preparing assay inocula were grown on medium M-5 agar slopes at 30°. Usually the growth from one slope was suspended in 2 ml. sterile water and 0·1 ml. inoculated into each assay tube. The tubes were shaken continuously during incubation for 48 hr. at 30°. The relative growth was read as optical density.

Thiamine concentration of the material under study was established, known amounts of thiamine added, and the microbiological assays performed. Results are shown in Table 5. The lower curve in Fig. 8 is the standard curve for reference. In most instances about 95% of the thiamine added was found. In two tests only were the amounts recovered appreciably less than those added, and here the total thiamine found was 8 mμg./10 ml. or less. Recoveries in the range of 10–64 mμg. thiamine/10 ml. seemed excellent. The data indicate that the error in this type of experiment probably would not exceed ±10%. None of the constituents of the materials tested interfered appreciably with the measurement of the thiamine added.

Table 5. *Recovery experiments using* Flavobacterium aquatile *to measure known amounts of thiamine.HCl added to various materials*

		Amount of thiamine.HCl (mμg.)				
Substance	Sample no.	Initially present*	Added	Total theoretically present	Total assayed*	% recovery†
Vitamin B complex pills	1	2	0	2	—	—
	2	2	5	7	5	71
	3	2	30	32	31	97
	4	14	0	14	—	—
	5	14	5	19	19	100
	6	14	30	44	47	107
Commercial wheat cereal	1	4	0	4	—	—
	2	4	5	9	8	89
	3	4	30	34	38	112
	4	34	0	34	—	—
	5	34	5	39	40	103
	6	34	30	64	62	97
Commercial tomato juice	1	4	0	4	—	—
	2	4	10	14	13	93
	3	4	30	34	35	103
	4	7	0	7	—	—
	5	7	10	17	16	94
	6	7	30	37	37	100
Commercial wheat germ	1	5	0	5	—	—
	2	5	5	10	10	100
	3	5	40	45	48	107
Human urine	1	3	0	3	—	—
	2	3	5	8	6	75
	3	3	40	43	42	98
	4	32	0	32	—	—
	5	32	5	37	36	97
	6	32	40	72	63	88

* Average of duplicate determinations.
† Based upon total thiamine content.

DISCUSSION

Lactobacillus fermenti appears to be the most widely used organism for tube microbiological assays of thiamine. *L. fermenti* requires a complex basal medium and may show some vagaries in its response to thiamine. These circumstances have led to improved methods for using *L. fermenti* (Maciasr, 1957; Banhidi, 1958) as well as to a search for micro-organisms better suited to the microbiological assay for thiamine. *Kloeckera brevis* (Hoff-Jorgensen & Hansen, 1955) offers some advantages as compared with *L. fermenti* but the basal medium required is complex, its preparation tedious in routine work, and the organism cannot be used to measure the total thiamine present in natural materials unless combined thiamine first is liberated enzymically. *L. viridescens* seems well suited to routine microbiological assays for thiamine; Deibel *et al.* (1957) showed that a simply and easily prepared basal medium may be used and that the growth responses to thiamine are reproducible. Also, this organism responds equally well to thiamine and to thiamine pyrophosphate. The growth responses of *L. viridescens* are related quantitatively to thiamine concentrations in the range 0–100 mμg./10 ml. medium. This range of concentrations is approximately 10-fold greater than that effective for *K. brevis* (Hoff-Jorgensen & Hansen, 1955). The growth responses of *K. brevis* and *L. viridescens* are not influenced by cultural conditions nor by the composition of basal assay media to the same extent as are the growth responses of *L. fermenti.*

The studies reported here indicate that *Flavobacterium aquatile* strain Taylor offers most of the advantages shown by *Kloeckera brevis* and *Lactobacillus viridescens* in microbiological assays for thiamine, and has other characteristics to recommend its use. The basal assay medium is less complex than those recommended for *K. brevis* or *L. viridescens*, especially supplements such as yeast extract are not required. No known growth factors other than thiamine need be added to the basal medium; no strains of *F. aquatile* were found which grew without added thiamine; the requirement for thiamine (or thiamine pyrophosphate) appears to be specific; the response to thiamine is reproducible; interference with the growth response to thiamine by specific compounds or those included in the natural materials studied was minimal. The concentrations of thiamine which may be measured with *F. aquatile* are comparable to those measurable by *L. viridescens*. Other useful attributes of *F. aquatile* in its use for the assay of thiamine include the ease with which the bacterium may be grown and the inocula prepared, and that the results of the assay are not critically influenced by amount of inoculum used, when the incubation period is 48 hr. Stock cultures of the bacterium may be transferred as infrequently as once a month and remain adequate sources for the inoculation of the assay media. Maximum growth responses to thiamine do require inocula prepared from stock cultures which are transferred each week. It would appear that *F. aquatile* is well suited for the measurement of the thiamine content of many natural materials.

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Preferential Compatibility in *Streptomyces violaceoruber*

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SUMMARY

A compatibility system, or system of relative mating potency, has been found among mutants of *Streptomyces violaceoruber* (*S. coelicolor*). Certain combinations of growth factor-dependent mutants failed to form nutritionally independent (prototrophic) recombinants. Incompatibility was not the result of inability of identical mutant genes to interact, failure of hyphae to fuse, or inhibition of prototroph development by incompatible mixtures. No infective or diffusible factors were found which induced or suppressed fertility. The yield of recombinants was affected by the relative growth rates of the two parental types, the time elapsed between the planting of each parent and the relative and absolute numbers of the parents. Sporogenesis was not a prerequisite for recombination.

INTRODUCTION

Gene recombination in *Streptomyces violaceoruber* (*S. coelicolor*) was reported by Sermonti & Spada-Sermonti (1955, 1956). They isolated nonparental types from mixed growth of complementary multiple auxotrophic mutants of *S. violaceoruber*. Similar systems were reported in *S. griseus* (Bradley & Lederberg, 1956), in *S. violaceoruber* (Bradley, 1957; Hopwood, 1959), in *S. violaceoruber* and *S. fradiae* (Szybalski & Braendle, 1956), in *S. rimosus* (Alikhanian & Mindlin, 1957), and in *S. griseoflavus* (Saito, 1958). Although the recombinational mechanism has been studied extensively, little consideration has been given to preferential mating. The occurrence of incompatibility in Streptomyces was expected inasmuch as incompatibility reactions have been established in several species of fungi and bacteria. For example, a non-filterable contagious factor that could be transmitted to other cells by direct contact was found to be responsible for fertility in *Escherichia coli* strain K-12 (Lederberg, Cavalli & Lederberg, 1952). In *Neurospora crassa* heterokaryotic incompatibility was manifested primarily in the vegetative hyphae (Garnjobst & Wilson, 1956). Hyphal fusion in non-fertile combinations of *N. crassa* was followed by a series of visible changes involving irreversible destruction of fusing cells. At first, all strains of *S. violaceoruber* tested interacted to form prototrophic progeny. Subsequently, growth factor-dependent mutants unable to interact with other variants were found and evidence for the existence of a compatibility system controlling recombination in *S. violaceoruber* was presented (Bradley & Anderson, 1958). The purpose of this study was to determine and analyse the genetic and environmental factors controlling recombination in *S. violaceoruber*.

Glossary

Prototroph. An organism which grew on minimal medium composed only of glucose, inorganic salts and water.

Auxotroph. An organism which did not grow on minimal medium unless supplemented with one or more specific growth-factors.

Recombination. The process by which two strains interacted to produce offspring phenotypically unlike the parents. A recombinant might be either prototrophic or auxotrophic.

Compatibility or *fertility.* The experimentally observed production of recombinants.

Heterokaryon. An organism having genetically different nuclei within a common cytoplasm.

Sterility. Inability to detect production of recombinants.

Streptomyces violaceoruber. This is a species which has often been erroneously designated as *Streptomyces coelicolor*. *S. violaceoruber* forms spirals, utilizes L-rhamnose and raffinose, and produces blue pigment on many media. *S. coelicolor* forms straight spore chains, does not utilize L-rhamnose and raffinose, and does not produce blue pigment on most media.

METHODS

The strains used in this study were auxotrophic (growth factor-dependent) mutants derived from a single wild-type strain of *Streptomyces vinaceus* and three wild-type strains of *S. violaceoruber* (Table 1). These latter organisms were previously designated *S. coelicolor*; Kutzner & Waksman (1959), however, pointed out recently that the correct epithet is *S. violaceoruber*.

Stock cultures were maintained on complete medium agar of the following composition. Solution I: glucose, 20 g.; Difco yeast extract (Difco Laboratories, Inc., Detroit, Michigan, U.S.A.) 1 g.; Difco Casamino acids, 1 g.; Difco agar, 15 g.; $MgSO_4.7H_2O$, 0·5 g.; deionized water, 500 ml. Solution II: KNO_3, 2 g.; Na_2HPO_4, 2 g.; deionized water, 500 ml. The composition of minimal medium was identical with that of complete medium except the former lacked yeast extract and Casamino acids. In the preparation of both complete medium and minimal medium, the two solutions were autoclaved separately and mixed before the media were dispensed. The complex medium was composed of: Difco peptone, 5 g.; Difco yeast extract, 3 g.; glucose, 1 g.; Difco Casitone, 1 g.; Armour beef extract, 2 g.; Difco agar, 15 g.; deionized water, 1 l. Comparable liquid media were made by omitting the agar. All media were autoclaved at 121° for 20 min.

Auxotrophic mutants were detected by the following method. Spores which survived ultraviolet irradiation (99 % kill) were cultured on complete medium; the resulting growth was transferred to minimal and complete media by replica plating (Lederberg & Lederberg, 1952); colonies which yielded growth on complete medium but not on minimal medium were tentatively designated as growth factor-dependent variants. The auxanographic technique was employed in characterizing all mutants. Susceptibility of natural variants or selected variants to actinophage P7 was also used as a genetic marker; phage-resistant strains were selected by planting 10^6–10^8

plating units of the phage-sensitive organism on complex medium seeded with 10^7–10^9 actinophage particles. Colonies arising on these plates were picked and streaked on complex medium containing an excess of actinophage to verify resistance. Susceptibility to actinophage was determined by the cross-streak technique: a single streak 8 cm. long, of phage suspension (0·01 ml. of a 10^8 phage particles/ml. suspension) was made on complex medium agar and allowed to dry; streptomycetes to be tested were cross-streaked against this single phage streak. Failure of the test organisms to grow at the intersections indicated susceptibility to the phage. This method permitted rapid classification of a large number of progeny. Morphological differences inherent in the strains, such as colonial texture and pigmentation, were noted but not considered reliable genetic markers.

Table 1. *Principal stocks*

Strain	Origin	Phenotype*
S199	*S. violaceoruber* Nature, Wisc. Soil Sample	Prototrophic P7^s
S202	S199	Cystine$^-$ Proline$^-$ P7^s
S203	S199	Uracil$^-$ arginine$^-$ P7^s
S204	S199	Methionine$^-$ P7^s
Sermonti 1	*S. violaceoruber* G. Sermonti, Rome, Italy	Prototrophic P7^s
S205	Sermonti 1	Histidine$^-$ P7^s
Sermonti 23	Sermonti 1	Methionine$^-$ histidine$^-$ P7^s
Sermonti 30	Sermonti 1	Nicotinic acid$^-$
S16	*S. violaceoruber* NRRL-B-1257 U.S. Dept. Agric. Peoria, Ill.	Prototrophic P7^r
S206	S16	Arginine$^-$ P7^r
S207	S16	Hydroxy-proline$^-$ P7^r
S125	S16	Methionine$^-$ P7^r
S208	*S. vinaceus* Nature, Minn. Soil Sample	Prototrophic P7^r
S216	S208	Aspartic acid$^-$
S217	S208	Glutamic acid$^-$
S229	S207	Arginine$^-$ alanine$^-$ P7^r
S230	S207	Arginine$^-$ glutamic acid$^-$ P7^r

* Abbreviations: P7^s denotes susceptibility to actinophage P7; P7^r denotes resistance to actinophage P7; prototrophic denotes growth-factor independence; cystine$^-$ proline$^-$, etc., denote requirements for cystine and proline, etc., for growth.

Inocula for crossing experiments were prepared by harvesting aerial hyphae from stock plate cultures and suspending these hyphae in sterile deionized water. Preparation of inocula of mutants producing little or no aerial hyphae was more difficult because it was necessary to use vegetative mycelia. Potter–Elvehjem tissue homogenizers were used to reduce coarse suspensions of vegetative mycelia to homogeneous suspensions.

Recombinants were obtained by seeding approximately 10^5 plating units of each parent together on complex medium, incubating these mixed cultures at 30° for 6–14 days, and transferring samples to minimal and complete media by replica plating. Mixed growth was transferred to complete medium in order to verify the viability of both parental strains in the mixture and to provide an estimate of the relative growth of the parents on the crossing medium. Replica plates were scored for prototrophs after incubation at 30° for 3–5 days. In addition to their ability to grow on minimal medium, prototrophic colonies grew faster and produced greater quantities of aerial hyphae on all media than did the parental strains.

RESULTS

Compatibility system

Eleven auxotrophic mutants of *Streptomyces violaceoruber* were grown in most of the possible pairwise combinations on complex medium agar. Strain S207 (a derivative of wild-type strain S16) was not fertile with strains S202, S203 and S204 (descendants of wild-type strain S199) or with strains Sermonti 23 and Sermonti 30, which were derived from wild-type strain Sermonti 1 (Table 2). Strain S207 was fertile, however, with strains S125 and S206, which were derived from wild type strain S16. These results, and results of other crosses, supported the hypothesis that the closer the relationship between two organisms the higher was the fertility. This was not always true, however, for strain S125 produced more recombinants with strains S202 and S203 than with strains S206 or S207. Derivatives of *S. vinaceus* strain S208 were sterile in combination with all *S. violaceoruber* strains so far as they were tested, and therefore provided no information about this incompatibility system.

Table 2. *The compatibility system*

		Sermonti										
	S207	23	30	S216	S217	S206	S125	S229	S230	S204	S203	S202
S205	−	+	+	−	−	+	++	*	*	++	++	+++
S202	−	+	++	−	−	+	++++	*	*	++	++	
S203	−	++++	++	−	−	+	++++	*	*	++		
S204	−	++	++	−	−	+	+++	*	*			
S230	*	*	*	*	*	†	*	++				
S229	*	*	*	*	*	†	*					
S125	+	++	+	−	−	+++						
S206	+++	+	+	−	−							
S217	−	*	*	++								
S216	−	*	*									
Sermonti 30	−	++										
Sermonti 23	−											

Two complementary auxotrophic strains were grown together on complex medium at 30°. After 5–14 days, a sample of mixed growth was transferred to minimal medium by replica plating. Prototroph production was rated as:

+ = 5–25 prototrophs/plate.
++ = 10–50 prototrophs/plate.
+++ = 30–200 prototrophs/plate.
++++ = over 200 prototrophs/plate.
* = not tested.
† = not testable because these strains have a common metabolic defect.
− = no prototrophs produced.

Mechanism of incompatibility

It was conceivable that mixed growth of incompatible strains inhibited growth of prototrophs. To test this possibility, fragmented aerial hyphae from prototrophic wild-type strain S199 were added to mixed inocula of strains S205 and S207 (an incompatible combination) and the mixture was plated on complex medium. After incubation at 30° for 8 days, a sample of mixed growth was transferred to minimal medium by replica plating in an attempt to recover the S199 prototrophs added originally. Prototrophic colonies of strain S199 were clearly visible 5 days after incubation of the replica plates at 30° (Table 3). It was concluded that mixed growth of strains S207 and S205 did not inhibit the development of S199 prototrophs; therefore, it was unlikely that suppression of prototroph development by non-fertile combinations was responsible for incompatibility.

Table 3. *Recovery of S*199 *prototrophs from mixed growth of S*207 + *S*205

Inoculum size in plating units			Average number of recovered prototrophs (S199) per plate
S207	S205	S199	
5×10^6	5×10^6	10^3	230
5×10^6	5×10^6	10^2	22
5×10^6	5×10^6	10	2
5×10^6	5×10^6	0	0
5×10^6	0	10^2	20
0	5×10^6	10^2	18
0	5×10^6	0	0
5×10^6	0	0	0

Aerial hyphae from prototrophic wild-type strain S199 were added to mixed inocula of S205 and S207 (an incompatible combination) and the mixture was plated to complex medium. After incubation at 30° for 8 days, a sample of mixed growth was transferred to minimal medium by replica plating in an attempt to recover the S199 prototrophs added originally. Replica plates were scored for the presence of S199 colonies after incubation for 5 days at 30°.

A microscopic search for hyphal fusion in crosses between mutants involved in the compatibility system was made, using a maximum total magnification of × 2000. To detect hyphal fusion, two mutants were grown in close proximity on complete medium. When hyphal tips of the two strains were close enough for conjugation, a small block of agar including parallel streaks of the organisms was cut from the plate and carefully melted beneath a coverslip on a glass microscope slide. Search for hyphal fusion was conducted only in areas where the positions of both mutants could be definitely determined. Hyphal fusion occurred as well in non-fertile combinations as in fertile combinations. Fusion of hyphae was observed in the fertile combinations S206 + S207, S206 + S204, S206 + S125 and S203 + Sermonti 23, and also in the non-fertile combinations S207 + S205, S207 + S204, and S207 + S203. Incompatibility, therefore, did not result from inability of hyphae of incompatible pairs to fuse, since hyphal fusion occurred both in compatible and incompatible combinations.

To test the possibility that fertile combinations might produce a filtrable fertility factor which would induce compatibility in sterile combinations, the fertile combination S206+S125 and the incompatible combination S205+S207 were grown in complex medium broth on opposite sides of a fritted glass filter. The partitioned cultures were incubated at 30° for 9–16 days and the liquid medium was exchanged across the filter daily by using suction from a water aspirator. Following incubation, the growth mixtures were removed from both sides of the filter, harvested by centrifugation, and washed twice with sterile deionized water. The mixtures were then suspended in sterile deionized water and transferred aseptically to tissue homogenizers for dispersion to provide homogeneous inoculative suspensions. To determine whether prototrophs were formed, samples of each of the mixed growth suspensions were plated to minimal and complete media. The compatible S206+S125 combination was fertile under these conditions, but the incompatible S207+S205 combination was sterile (Table 4).

Table 4. *Tests for a filtrable fertility factor*

Pair 1 and pair 2 separated by	Pair 1	Average number of prototrophs/ plate	Pair 2	Average number of prototrophs/ plate
Fritted glass	S206+S125	250	S207+S205	0
Cellophan	S206+S125 (bottom)	13	S207+S205 (top)	0
	S206+S125 (top)	5	S207+S205 (bottom)	0

The fertile combination S206+S125 and the incompatible combination S207+S205 were grown on opposite sides of a fritted glass filter or a cellophan membrane. Following incubation at 30°, samples of mixed growth from both sides of the fritted filter (or cellophan membrane) were transferred to minimal medium. Minimal medium plates were scored for prototroph production after incubation at 30° for 3–5 days.

Additionally, the fertile combination S206+S125 and the incompatible combination S207+S205 were grown on opposite sides of a cellophan membrane, with inocula of approximately 5×10^6 plating units of each parental strain. After incubation at 30° for 8 days, samples of mixed growth from each side of the membrane were suspended in deionized water, dispersed in a tissue homogenizer, and plated on minimal and complete media to determine prototroph production. All compatible pairs yielded prototrophs but incompatible combinations remained sterile. The results of this experiment further confirmed the hypothesis that fertile combinations did not produce filtrable factors capable of inducing fertility in incompatible combinations.

An infective factor, similar to the contagious F+ factor in *Escherichia coli* might control fertility in the compatibility system. To test this possibility, two complementary auxotrophic strains were grown in mixed culture on complex medium for 8 days at 30°. Then one or both of the parents were recovered from the mixed growth by transferring (replica plating) the mixed growth to minimal media supplemented with the specific nutritional requirements of the organisms being recovered. The parents, re-isolated after growth in a fertile combination, were then crossed to other strains to determine whether factors were acquired which modified ability to be successfully crossed or induced fertility in incompatible pairs. Non-fertile combinations, however, remained sterile even though one or both parents had been previously grown in fertile combinations (Table 5). The results, therefore, indicated that no contagious fertility factor was responsible for compatibility in *Streptomyces violaceoruber* and that prior association with a compatible partner did not increase the recombinational frequency in subsequent crosses, Conversely, Braendle, Gardiner & Szybalski (1959), found that complementary 'secondary parental isolates' yielded more heterokaryons/plate than 'primary parental isolates'.

Table 5. *Tests for a contagious fertility factor*

Combination	Average number of prototrophs/ plate
S207+S206 (S205)	23
S207+S205 (S206)	0
S205+S206 (S207)	26
S205+S207 (S206)	0
S205+S207 (S125)	0
S205 (S206)+S207 (S125)	0

Symbolism: S206 (S205) denotes that S206 had been grown in combination with S205 and then re-isolated.

Two complementary auxotrophic strains were grown together on complex medium at 30°. After 8 days, one or both of the parents were recovered from the mixed growth by replica plating the mixed growth to minimal media supplemented with the specific nutritional requirements of the organisms being recovered. Recovered strains were crossed to other strains as desired by growing the two organisms in mixed culture on complex medium at 30°. After 8 days, samples of mixed growth were transferred to minimal medium by replica plating to determine prototroph production.

Table 6. *Effect of age of the mixed culture on prototroph production*

	Average number of prototrophs/plate	
Days of incubation	S206+S125	S203+Sermonti 23
1	0	0
2	0	0
3	20	93
4	19	106
5	27	103
6	31	131
7	30	125
8	33	142
9	28	136
10	40	129

Pairwise mixtures of complementary auxotrophic strains were grown on complex medium at 30°. After 24 hr. and daily thereafter for 10 days, duplicate samples of mixed growth were transferred to minimal medium by replica plating. Replica plates were scored for prototroph production after 3–5 days incubation at 30°.

The fertile combinations S206+S125 and S203+Sermonti 23 were grown on complex medium agar to determine the minimum time interval between mixing of the mutant strains and detection of prototrophic progeny. Also, it was desirable to know whether the total number of prototrophs produced from a fertile combination increased after the time of first detection. Twenty-four hours after mixing the mutants, and daily thereafter for 10 days, duplicate samples of mixed growth were transferred to minimal and complete media by replica plating to determine prototroph production. Prototrophs were not detected until the mixed cultures had been incubated for 3 days; from the third day of incubation to the tenth day of incubation, the quantity of prototrophs produced remained constant or increased slightly (Table 6).

Effect of inoculum size on prototroph production

Prototroph production was also affected by the inoculum size of each of the auxotrophic mutants involved in a cross and by the relative growth rates of the auxotrophic parents. In the Sermonti 23 + S 203 combination, only *c.* 2 prototrophs/plate were produced when the inoculum of the former strain (approximately 5×10^6 plating units) exceeded greatly the inoculum of the latter strain (approximately 5×10^2 plating units). No prototrophs were produced when the inoculum of strain S 203 greatly exceeded the inoculum of strain Sermonti 23. An average of 10 prototrophs/plate were formed when the inocula of both parents were approximately 5×10^2 plating units, and an average of 23 prototrophs/plate were produced when the inocula of both parents were approximately 5×10^6 plating units.

Decrease in prototroph production caused by a difference in the growth rates of the auxotrophic parents could be countered in most cases by allowing the slow growing parent to grow for 1–2 days before addition of the fast growing parent. When the growth rates of both parents were about equal, addition of one of the parents 1–3 days after the other parent did not appreciably affect prototroph production. When addition of the inoculum of the second parent was delayed longer than 3 days, no prototrophs were formed. These results indicated either that established growth no longer had the ability to form prototrophs or that an antibiotic was produced which killed the delayed inoculum. The latter alternative was disproved by demonstrating that both parental types could be recovered.

Yield of recombinants from aerial hyphae and vegetative hyphae

It was of interest to ascertain whether prototrophic recombinants could be recovered from both aerial and vegetative hyphae and to determine which of these sources produced the greatest quantity of prototrophs. As a representative fertile combination, strains S 203 and Sermonti 23 were grown in mixed culture on complex medium at 30° for 10 days. Suspensions of aerial and vegetative hyphae were then prepared from the mixed growth and samples of these suspensions were plated on minimal and complete media. Relatively homogeneous suspensions for inoculations were prepared by thoroughly grinding the coarse hyphal suspensions in sterile Potter–Elvehjem tissue homogenizers. The number of plating units/ml. of the aerial and vegetative inoculum suspensions were calculated from counts obtained with a Petroff-Hauser bacteria counter. Plates were scored for prototroph formation after incubation at 30° for 4 days. An average of $2 \cdot 5 \times 10^{-5}$ prototrophs/plating unit of mixed growth were obtained from the aerial hyphae of the Sermonti 23 + S 203 combination and an average of $5 \cdot 6 \times 10^{-5}$ prototrophs/plating unit were produced from the vegetative hyphae. The results of these experiments showed that in the compatible combination S 203 + Sermonti 23, approximately twice as many prototrophs were produced in the vegetative hyphae as in the aerial hyphae, per plating unit of mixed growth.

Phage susceptibility tests on prototrophic recombinants

Prototrophic progeny from several combinations in the compatibility system were tested for susceptibility to actinophage P7. Phage susceptibility of 106 prototrophic colonies from crosses between strains S 206 and S 205, and of 115 prototrophic colonies from crosses between strains S 206 and S 204, was determined using the cross-streak technique. In both crosses, susceptibility to actinophage P7 could segregate since strain S 206 was resistant to phage P7 and strains S 205 and S 204 were sensitive. All prototrophs tested from the S 205 (P7 sensitive) × S 206 (P7 resistant) cross and the S 204 (P7 sensitive) × S 206 (P7 resistant) cross were found to be sensitive. These results suggested that the recombinants were heterokaryons, heterozygous diploids, or haploid gene recombinants in which actinophage susceptibility was linked closely to a selected character.

DISCUSSION

Certain nutritionally dependent mutants of *Streptomyces violaceoruber* in pairwise mixtures failed to form growth factor-independent prototrophic colonies (Bradley & Anderson, 1958). Failure of these combinations to produce prototrophs was not the result of inability of identical mutant genes determining nutritional requirements to interact, because diverse markers were used. Moreover, two strains, each unable to interact with a third strain, were compatible with each other, a situation which would have been impossible if the genes preventing heterokaryon formation were identical. Therefore a specific compatibility system exists which controls recombination. The possibility that prototroph development was inhibited in an incompatible combination was ruled out by the successful recovery of added strain S 199 prototrophs from the incompatible S 207 + S 205 mixture, following incubation. Hyphal fusion occurred in non-fertile as well as in fertile combinations, so inability of hyphae of incompatible pairs to fuse was not responsible for incompatibility. The barrier to compatibility might operate by preventing nuclear exchange or by preventing establishment of balanced nuclear ratios. The former hypothesis seems probable in view of the finding that fusion of hyphae in non-fertile combinations of *Neurospora crassa* is followed by destruction of fusing cells (Garnjobst & Wilson, 1956). The difficulty of cytological studies following hyphal fusion in sterile combinations of *S. violaceoruber* prevents positive comparison, however, of the reaction occurring in *S. violaceoruber* with the irreversible reaction occurring in *N. crassa*. No filtrable or infective fertility factors were demonstrated in the compatibility system described here.

The strains of *Streptomyces violaceoruber* studied here can be divided into three groups (designated A, B, and C) on the basis of their compatibility behaviour. Strains in group B produce prototrophs in combination with all other mutants of *S. violaceoruber* not having the same metabolic defect. Mutants belonging to group A are compatible with members of group B but not with members of group C. Groups A and B include mutant strains derived from wild type strain S 16. Group A is exemplified by strain S 207 and group B by strain S 125. Group C, which contains mutants derived from wild-type strains Sermonti 1 and S 199, is typified by strain S 203. The general pattern of the compatibility system controlling recombination in *S. violaceoruber* may be summarized as follows: A + B, B + C, A + A, B + B, and

C + C are fertile; A + C is not fertile. The data indicate that two or more factors may control heterokaryotic compatibility in *S. violaceoruber* The genotypes of A, B, and C can be symbolized as a_1b_1, a_1b_2, and a_2b_2, respectively. For mating to occur, the two strains involved must carry identical factors at one or both of the two loci. A fourth genotype a_2b_1, which has not yet been discovered, is predicted by this hypothesis. Accordingly, this strain should mate with A and C but not with B.

The compatibility described in this study represents interaction between three wild type strains of *Streptomyces violaceoruber* of different origin. Hence recombination was demonstrated between the derivatives of strains of diverse origin as well as between derivatives of a single wild type strain. These results differ from the observations of Braendle & Szybalski (1957) who reported that all crosses between mutants of diverse origin, whether or not the parental strains were classified in the same species, were non-fertile, i.e. no recombinants were formed. Crosses between mutant derivatives of *S. vinaceus* and mutants derived from each of the wild type strains of *S. violaceoruber* were unsuccessful, but a cross between two mutants derived from the same strain of *S. vinaceus* was fertile (Table 2).

All incompatible reactions in the compatibility system involve *Streptomyces violaceoruber* strain S207, a descendant of wild-type strain S16. Strain S207 was not fertile with derivatives of wild-type strains S199 (isolated from Wisconsin soil sample) and Sermonti 1 (provided by G. Sermonti). Strain S207 was fertile, however, with other derivatives of strain S16, from which strain S207 itself was derived. These and other results supported the hypothesis that the greater the similarity between two organisms, the higher the fertility. Exceptions to this hypothesis, however, are exemplified by the high fertility of crosses between strain S125 (a derivative of S16) and mutants derived from wild-type strains S199 and Sermonti 1.

Fertile combinations of auxotrophic mutants of *Streptomyces violaceoruber* must be grown on complex medium for a period of at least 3 days before prototrophs can be detected. It is not known whether this lag represents a delay in hyphal fusion or a delay following hyphal fusion. From the third day of incubation to the tenth day of incubation the quantity of prototrophs produced remained fairly constant. Maximum prototroph production was reached on about the fifth day. Both aerial hyphae and vegetative hyphae yielded prototrophic recombinants. The vegetative hyphae produced approximately twice as many prototrophs as the aerial hyphae per plating unit of mixed growth. This established that sporogenesis was not a prerequisite for recombination.

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Structure of the Mate-Killer (mu) particles in *Paramecium aurelia*, stock 540

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SUMMARY

The mate-killer (mu) particles in the cytoplasm of *Paramecium* aurelia, stock 540, variety (syngen) 1, were studied by: Feulgen-staining; phase-contrast microscopy; fluorescence under ultraviolet irradiation after staining with acridine orange; electron microscopy (including the 'silver-Feulgen' technique). The appearance of the particles following treatment with DNAase and RNAase was observed. It was found that the mu particles were capsulated rod-shaped structures, 2–10 μ long and about 0·3 μ in diameter (excluding the capsule); they appeared to reproduce by transverse fission without formation of cross-walls. There was an external double membrane and the internal material consisted largely of DNA, which was not limited to particular zones but spread throughout the interior of the particle. RNA was also present. The relationships of the particles to other micro-organisms is discussed, and it is concluded that they do not differ from bacteria in any important respect.

INTRODUCTION

Ever since the discovery of the 'killer' strains of *Paramecium aurelia* by Sonneborn (1938) there has been discussion about the nature and homologies of the cytoplasmic kappa particles responsible for the peculiar killing properties of these paramecia. At one time kappa particles were considered to be gene-like elements in the cytoplasm or plasmagenes (Sonneborn, 1947), but this view became less plausible as knowledge of the particles increased. For example Preer (1948) showed that they could be stained and seen with the light microscope, and were as large as some bacteria. Moreover, Sonneborn (1948) showed that at least one kind of kappa particle could be experimentally transmitted from one paramecium to another by infection through the external medium. These and other findings strengthened the belief, which had been firmly held all along by certain critics (e.g. Altenburg, 1946), that the particles were to be regarded as symbiotic micro-organisms rather than plasmagenes. Their exact position remains, however, uncertain, as can be seen from the discussion of Preer & Stark (1953) and the recent exhaustive review of Sonneborn (1959).

Characterization of a micro-organism would ideally require a thorough description of both morphological and physiological properties, but since kappa and similar particles cannot as yet be grown anywhere except within the cytoplasm of a paramecium, we are restricted to morphological and cytochemical studies. In this paper we give an account of the structure of one type of cytoplasmic particle called mu, occurring in *Paramecium aurelia*, stock 540 (variety or syngen 1). This stock was originally collected in Mexico, and was kindly made available to us by Dr T. M. Sonneborn. It is a mate-killer having the same general properties as the mate-killers earlier described by Siegel (1953) in variety 8. As found there, when conjugation between a mate-killer and a normal (sensitive) organism takes place, nuclear exchange proceeds normally, but one half of the ex-conjugants (those which receive cytoplasm from the sensitive conjugant) die after two fissions or less, whilst the other half (which receive cytoplasm, and hence mu particles, from the mate-killer conjugant) give rise to fully viable clones. The mechanism causing the death of the sensitive organisms is unknown, but it has been shown by Siegel (1954) that contact of the surfaces of mate-killer and sensitive paramecia for a minimum of about 2 hr. is required. Exchange of the internal contents, either nuclei or cytoplasm, is not an essential part of the killing process. A brief account of the stock 540 mate-killer, including some genetical information, was given previously (Beale, 1957). Out of 60 stocks of *P. aurelia* variety 1 collected from many different parts of the world, stock 540 is the only mate-killer.

METHODS

Cultures. The paramecia were grown on baked lettuce or dried grass infusions containing *Aerobacter aerogenes*. Abundant mu particles were obtained by allowing the cultures of paramecia to starve for 3–4 days at room temperature in dilute buffered saline. Under such conditions the particles continue to multiply whilst the paramecia do not. The ciliates were concentrated by drawing the culture fluid through a Berkefeld candle, and subsequent centrifugation of the organisms from the fluid which remained behind.

Light microscopy. The fixatives most commonly used were methanol + acetic acid (3:1), and 1 % (w/v) osmic acid buffered to pH 7·2. Time of fixation was 1 hr. For Feulgen staining the material was hydrolysed with N-HCl for 5 min. at 60°, and stained for $1\frac{1}{2}$ hr. (whole mounts) or $2\frac{1}{2}$ hr. (sections) at room temperature in dilute buffered saline. Azure A staining (see DeLamater, 1951) was by a procedure essentially the same as for the Feulgen procedure, except that in place of Feulgen reagent, staining was with azure A for 3–4 hr. at room temperature. Paraffin sections were cut at 2–7 μ thickness.

Preparations of mu particles for phase-contrast observations were made by gently crushing a few living paramecia on a slide with a coverglass and observing the particles directly (method of Preer & Stark, 1953).

For the demonstration of translucent capsules surrounding the particles, a method based on that described by Duguid (1951) was used. A dense suspension of paramecia was crushed between a slide and coverslip. The coverslip was then removed and placed on a drop of water on a clean slide. Indian ink was infiltrated into the preparation and the particles which adhered to the coverslip were observed by phase-contrast microscopy, with an oil-immersion objective.

Fluorescence of particles stained with acridine-orange was studied by a technique based on that described by Anderson, Armstrong & Niven (1959). The particles on a slide were washed with 0·01 M-acetate buffer (pH 5), stained for 2 hr. with acridine orange (2·5 mg. in 1 ml. of the same buffer), thoroughly washed with buffer, and examined under ultraviolet (u.v.) radiation under the microscope with a dark ground condenser (Zeiss). An Osram high-pressure mercury vapour lamp (type HBO 200) was used as source of u.v. radiation.

Electron microscopy. The preferred fixative was 1 % (w/v) osmic acid, buffered to pH 7·2 with veronal + acetate at 1/9 the concentration recommended by Palade (1952). For special purposes methanol + acetic acid (3:1) was also used as fixative. The time of fixation was 1 hr. at room temperature, after which the suspensions of whole paramecia were dehydrated, embedded in methacrylate and sectioned at about 250 Å. on a Porter-Blum Servall microtome. A Siemens Elmiscope I electron microscope was used.

Enzyme treatments. (*a*) *DNAase.* After fixation of whole paramecia with osmic acid or methanol + acetic acid and thorough rinsing in water and buffer (0·003 M-tris buffer at pH 7·6; 0·02 M-$MgSO_4.7H_2O$), the suspensions of organisms were divided into two parts (tris buffer, even though it causes considerable distortion, was used in place of phosphate buffer here since a precipitate formed when the latter is used with the DNAase preparation containing $MgSO_4$). One part was treated with a solution of crystalline DNAase (Nutritional Biochemicals Corp.; 1 mg./10 ml. buffer) for 2 hr. at 37·5°. The second part was left in buffer for the same time and at the same temperature, as a control. Following DNAase treatment samples were stained by the Feulgen method and examined in the light microscope to check the effectiveness of the digestion; the remainder were prepared for electron microscopy as described above.

(*b*) *RNAase.* After fixation with methanol + acetic acid (3:1) and thorough rinsing in 1/10 Ringer solution buffered to pH 6·8 with 0·004 M-phosphate, the suspensions of whole paramecia were treated with a 1 % (w/v) solution of crystalline RNAase (Light) for 3 hr. at 37°. Effectiveness of the digestion was checked by staining samples by the methyl-green pyronin method, and observing with the light microscope the absence of pyronin-stained material in the cytoplasm.

'*Silver-Feulgen*' *method* (based on a technique of Bradfield, 1954; see also Jurand, Deutsch & Dunn, 1959). After osmic fixation for 1 hr. and hydrolysis in N-HCl at 60° for 5 min. the material was washed with distilled water until no reaction took place with a 3 % (w/v) $AgNO_3$ solution, then treated for 1–3 hr. at 40–48° with a 1·5 % (w/v) aqueous solution of hexamethylenetetramine containing 0·25 % (w/v) $AgNO_3$ buffered with borate to pH 8·3. The paramecia were then washed in 1·5 % (w/v) hexamethylenetetramine, rinsed with water, then with 5 % (w/v) sodium thiosulphate and again with water. The material was then dehydrated and embedded in methacrylate.

RESULTS

Light microscope observations

Feulgen and azure-A staining. In mounts of whole paramecia the mu particles appeared as clouds of stained material (Pl. 1, fig. 2*a*); usually individual particles could not be distinguished. When the paramecia were sectioned, the individual particles were seen more clearly (Pl. 1, fig. 1) and were then revealed as Feulgen-positive rod-shaped structures, with no internal differentiation. After 3–4 days starvation of the paramecia, the particles were extremely numerous and occupied almost the entire cytoplasm. With less severe starvation of the paramecia the particles were aggregated in one or two large colonies, in a manner similar to that described previously by Siegel (1954) for the mu particles in other stocks. Following DNAase treatment of mate-killer paramecia, no Feulgen-positive material remained in the cytoplasm, nor, of course, in the nucleus (Pl. 1, fig. 2*b*). (The darker material in Pl. 1, fig. 2*b*, is due to browning produced by osmic acid, not to the Feulgen reaction.)

Phase-contrast observations. The mu particles were not seen inside living unstained paramecia, but when the latter were gently crushed, causing the contents to flow out, the particles became easily visible in phase contrast, as previously recorded for the kappa particles in other stocks by Preer & Stark (1953). Stock 540 mu particles became characteristically orientated flat on the glass slide (Pl. 1, fig. 3*a*), and were not washed off with distilled water or culture fluid, nor were they removed when the coverslip was taken off, the fluid allowed to dry and the preparation re-wetted. Some of the particles likewise adhered to the coverslip. These treatments left the particles adhering to the glass surface, and it was therefore possible to obtain a homogeneous preparation of mu particles since most other cytoplasmic structures were washed away with water. Solutions of higher ionic strength (2 %, w/v, NaCl or N-HCl) caused the particles to become detached from the glass. As seen in phase contrast, the particles were usually about 2μ in length, but varied between 2 and 10μ. A constriction was frequently seen, but no internal structural details were discerned. The refractile bodies described by Preer & Stark (1953) in kappa particles were not present in the mu particles. It will be noticed in Pl. 1, fig. 3*a*, that the particles never appear to come into direct contact with one another, and this is a general rule. Even after DNAase treatment, which removed all Feulgen-positive material (this constitutes the bulk of the internal contents of the particles; see below) the appearance of the latter in phase contrast remained unchanged; and the same was found after RNAase treatment. The particles therefore possess a rigid envelope made of some substance other than nucleic acid. The presence of translucent material or capsules surrounding the particles was clearly shown by the indian ink technique (see Pl. 1, fig. 3*b*). Considerable variation in the thickness of the capsule was found in different particles, but it was commonly about $0{\cdot}2\mu$ thick.

Fluorescence following acridine-orange staining. Cells of higher organisms, and also bacteria, when stained with acridine orange at particular pH values and examined under u.v. radiation, show differential fluorescence of DNA- and RNA-containing regions (see Anderson *et al.* 1959). For example these authors report that, at pH 5, RNA fluoresced red and DNA green or greenish yellow in the bacteria *Salmonella typhi* and *Escherichia coli*.

Preparations of unfixed and of methanol + acetic acid fixed mu particles on glass slides obtained by crushing the paramecia were stained with acridine orange and examined under u.v. radiation. The fluorescence of the mu particles was always weak, notwithstanding use of longer and stronger staining procedures than those recommended by Anderson *et al.* For comparison we sometimes included in the same preparations bacteria (*Aerobacter aerogenes*) and cytoplasm and nuclei of Paramecium, and found that these fluoresced brightly as expected.

At pH 5 the mu particles fluoresced a pale yellowish colour. When they had been pretreated with DNAase and then stained with acridine orange, fluorescence was somewhat more orange, and when the pretreatment was with RNAase, the fluorescence became more greenish, than in the preparations not treated with enzymes. It is therefore concluded that the particles contained RNA and DNA, but the reason for the faintness of the fluorescence is not known. In any case no internal differentiation of RNA- and DNA-containing regions was demonstrated by this method.

Electron microscope observations

Ultra-thin sections of mu particles in the cytoplasm of osmic acid-fixed paramecia, as seen with the electron microscope, are shown in Pl. 1 and 2, figs. 4–8. The particles are seen to be bounded by a double membrane, have a diameter of about $0{\cdot}3\,\mu$, are usually quite straight, and contain material which appears more dense than the cytoplasm of the paramecia. Within the dense material there is an arrangement of irregular vacuole-like areas, within which dense granules are frequently seen. Sometimes a section was obtained which showed a rather regular row of these 'vacuoles', each one containing a dense central spot, but usually the pale regions were irregularly distributed and might be close against the external membrane. A single large vacuole was often found at one end of a particle (Pl. 2, fig. 8). In particles which showed a median constriction, and were apparently about to undergo transverse fission, no internal cross-wall was seen (Pl. 1, fig. 4). Around the outside of the particles a pale area was commonly (though not always) found, corresponding to the capsule as seen in phase contrast by the indian ink technique (see especially Pl. 1, fig. 6). (Mitochondria and other cytoplasmic inclusions showed no such surrounding pale area.) Where the particles were concentrated in one or two large groups in the cytoplasm, the latter appeared generally less dense in that region than where there were few or no particles. A number of normal paramecia (not mate-killers) were sectioned and examined in the electron microscope; none was found to contain the mu particles as described above.

Effect of enzyme treatment on appearance of particles in the electron microscope. In Pl. 3, figs. 9*b* and 10*b*, are shown preparations of paramecia treated with DNAase before embedding and sectioning. Pl. 3, fig. 10, shows material fixed with osmic acid (and washed with tris buffer) and Pl. 3, fig. 9, material fixed with methanol + acetic acid (and washed with phosphate buffer). In each case samples of DNAase-treated material were stained by the Feulgen method and confirmation was obtained that all Feulgen-positive material had been removed from the mu particles and from the macronucleus. Further, DNAase-treated material was stained with pyronin; it gave positive reactions. Thus it was confirmed that DNAase treatment removed DNA but not RNA, and that the fixatives used did not interfere with subsequent digestion by DNAase.

The DNAase-treated material was compared with controls which were treated according to the same procedure of fixation, washing in buffer, etc., but not treated with enzyme (Pl. 3, figs. 9*a*, 10*a*). This was important since the appearance of the particles in the electron microscope was markedly affected by such treatments, as can be seen by comparison of Pl. 3, figs. 9*a*, 10*a* with Pl. 1, figs. 4–6. We have never considered it worth while to make comparisons between preparations made at different times, since there is no doubt that slight variations in the kind of buffer, times and temperatures of washing, etc., have profound effects on the appearance of the material in the electron microscope. The effect of the DNAase treatment was clearly to remove the greater part of the electron-dense material from both osmic-acid-fixed and methanol + acetic-acid-fixed mu particles. Some electron-dense material remained round the outer margin and a few irregular granules within. In the methanol + acetic acid-fixed preparations, the contents of the particles were shrunken away from the membrane; DNAase treatment resulted in some swelling (Pl. 3, figs. 9*b*, 10*b*). From these observations it is concluded that most of the electron-dense material inside the particles contained DNA, which was not restricted to any central 'nuclear' region.

The effects of treatment with the RNAase preparation were also studied, and were quite clear in spite of the severe distortion brought about by the methanol + acetic acid fixation and rinsing in Ringer solution, etc. (see Pl. 4, fig. 13*a*). RNAase treatment resulted in the disappearance of the pale vacuole-like regions, so that the particles became uniformly opaque (Pl. 4, fig. 13*b*). A superficial resemblance may be noted between the particles in Pl. 4, fig. 13*b* (RNAase treated), and Pl. 3, fig. 9*a* (controls for DNAase experiment), but this is due to the fact that in Pl. 3, fig. 9*a*, the pale areas in the particles were much decreased in size on account of the tris buffer used. It is tentatively concluded that RNA is present in the 'pale' regions, and that the structure of the latter is destroyed by RNAase, allowing the electron-dense material to flow in. It is not excluded, of course, that RNA is present also in the electron-dense regions before treatment with RNAase.

'*Silver-Feulgen' reaction.* As described in the 'Methods' section, some mate-killer paramecia were hydrolysed with N-HCl and impregnated with silver nitrate. The idea behind this method is that products of hydrolysis of DNA (aldehydes), which re-colorize Schiff's reagent in the normal Feulgen technique, would also be expected to reduce silver nitrate to metallic silver, which could then be detected in the electron microscope. Some paramecia were therefore hydrolysed and impregnated with silver nitrate, then embedded, sectioned and observed in the electron microscope. The mu particles in these preparations were found to contain a massive electron-dense deposit—('silver granules'), especially in a zone just inside the outer double membrane, and to a lesser degree in the interior of the particles (Pl. 4, fig. 11). Other cytoplasmic structures such as mitochondria showed no such concentration though there was a background of electron-dense granules randomly distributed throughout (though much less dense than in the mu particles). The macronucleus, as expected, showed a dense accumulation of electron-dense granules. Unhydrolysed material when treated with silver nitrate in the same way showed no differential accumulation of silver granules in the mu particles or elsewhere, but merely a random deposition. That reduction of silver to the metallic form was in fact brought about by the hydrolysis products of DNA was shown by studying the effect of DNAase on preparations subsequently treated with silver nitrate. Pl. 4, figs. 12*a*, 12*b*, shows a comparison of DNAase-treated and control particles, and clearly reveals an almost complete absence of silver granules in the enzyme-treated particles, in spite of severe distortion of the structure of the particles due to the various chemical treatments.

DISCUSSION

From these observations it is concluded that the mu particles in *Paramecium aurelia* stock 540 (mate-killer) are rod-shaped structures varying in length between 2 and 10μ, width about 0·3μ, and surrounded by a double membrane and capsule. In a general way they resemble the kappa particles of other stocks of *P. aurelia* described by Dippell (1958) and Hamilton & Gettner (1958), but there are differences in detail. The mu particles studied here differ from the kappa particles by being longer and thinner, in lacking the refractile bodies found in a proportion of kappa particles (so-called 'brights'), and in the presence of a capsule. The kappa particles are more or less uniformly distributed throughout the cytoplasm whilst the mu particles form large clusters; it seems likely that this clumping is connected with the presence of the capsular material, since it will be remembered that the cytoplasm of paramecia is a fluid medium constantly circulating in the living organisms. The mu particles, like kappa particles, contain DNA and this substance is not confined within central 'nuclear' bodies, but spread throughout the particles. RNA is also present, as also reported for kappa by Dippell (1959*a*, *b*).

The mu particles resemble certain bacilli in size and shape and it is with these organisms that we should compare the particles most closely. The most striking difference is in the localization of the 'nuclear' material. With many bacteria, even though no chromosomes can be seen, the 'nuclei' are localized in a definite zone in the centre of the cell, containing Feulgen-positive material. In electron micrographs this zone appears less dense than the rest of the bacterial contents, but within this less dense zone extremely electron-dense material may sometimes be seen and the latter has been considered to be identical with the Feulgen-positive material seen in the light microscope (Maaløe & Birch-Andersen, 1956). These workers consider that in bacteria fixed with osmic acid or formaldehyde aggregates of DNA are objects which appear dark in electron micrographs. This would be in conformance with the results of our studies on the effect of treatment of the mu particles with DNAase, which led to a considerable decrease or elimination of electron-dense material within the particles in both osmic acid and methanol + acetic-acid-fixed material. What is unusual about the contents of the mu particles is that the DNA-containing material seems to occupy practically the whole of the interior of the particle, extending even to the external double membrane.

Concerning the 'silver-Feulgen' technique, several different interpretations are possible (as indeed is true also of the classical Feulgen reaction itself). We have found that some structures in paramecia which are unlikely to contain DNA, e.g. parts of the pellicle, may also cause a reduction to metallic silver in hydrolysed material. However, our results with DNAase treatment showed that the silver deposit within the mu particles was in fact due to the presence within them of DNA hydrolysis products. The fact that so high a density of silver granules was found just within the outer double membrane is not clearly understood but we assume is due to the reduction of silver ion immediately on entering the particle, so that a concentration gradient is set up. It seems unlikely that there is localization of DNA around the outside of the particles.

Doubtless objections could be raised against the various interpretations of individual cytochemical reactions which are described here, and particularly with regard to enzyme treatments of material examined by electron microscopy. However, when the results of all the tests are considered, a consistent picture is obtained. All the different techniques studied (Feulgen staining; fluorescence of acridine-orange-stained particles; DNAase treatment of particles observed in the electron microscope; the 'silver-Feulgen' technique) have given the same result, namely that the DNA in the particles is spread throughout them. This kind of distribution is not typical of many bacteria which have been examined cytochemically (as earlier noted by Preer & Stark, 1953). Nevertheless, it is known that the appearance of bacterial 'nuclei' is much affected by the nutritional and ionic conditions before and during fixation and staining (Robinow, 1956), and the habitat of the mu and kappa particles is after all very different from that of the saprophytic bacteria normally studied. The mu particles also differ from many bacteria in their structure during division. Sections of dividing bacteria often show a cross-wall developing between the two halves (see for example Bradfield, 1956); in the constricted mu particles, which one assumes are in the process of fission, no such cross-walls were seen. However, this distinction is unlikely to be critical and many bacteria may well be found to divide by constriction rather than transverse septum formation. Finally, the mu particles apparently lack the cytoplasmic membrane, distinct from the cell wall, often seen in electron micrographs of bacteria.

Whether the mu (and kappa) particles should be classified amongst the bacteria depends entirely on what definition is placed on that group. Doubtless the obligatory intracellular habitat of the particles has led to a nutritional situation where it is unnecessary for them to maintain the metabolic machinery of free-living forms. They would not require many of the enzymes located in the 'cytoplasm' of bacteria, and this might result in a greater proportion of the structure being occupied by nuclear material (as pointed out by Sonneborn, 1959). We therefore conclude that the mu particles are bacteria which have become adapted to life in the cytoplasm of paramecia.

We wish to thank Dr K. Deutsch for his collaboration with electron microscopy during the early stages of this work; also Drs T. M. Sonneborn and J. P. Duguid for criticism of the manuscript, and the last mentioned for drawing our attention to the indian ink technique. One of us (A. J.) is a Research Worker of the British Empire Cancer Campaign.

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EXPLANATION OF PLATES

Figs. 1–3. Light microscope preparations. Figs. 4–13. Electron microscope preparations. *mu*, mu particles; *ma*, macronucleus; *fv*, food vacuole; *cap*, capsule.

PLATE 1

Fig. 1. Paraffin section of *P. aurelia*, stock 540 mate-killer, fixed with osmic acid, hydrolysed with N-HCl, stained with azure A, examined in phase contrast.

Fig. 2*a*. Whole mount, fixed and stained as in fig. 1.

Fig. 2*b*. As fig 2*a*, but treated with DNAase before applying stain. The osmic acid causes some browning (dark in photograph), but the organism is completely unstained.

Fig. 3*a*. Unstained mu particles outside paramecium, flat on glass slide, observed in phase contrast. Note separation of particles.

Fig. 3*b*. Unstained mu particles lying flat on coverslip (or hanging vertically). Mounted in indian ink to show capsules. The indian ink particles are in Brownian movement, and produce grey background due to 10 sec. exposure of photographic plate.

Fig. 4. EM section through a mu particle, fixed with osmic acid, showing constriction.

Fig. 5. Longitudinal section through a long mu particle.

Fig. 6. Group of mu particles showing capsules in EM.

PLATE 2

Fig. 7. Comparison of mu particle with bacteria (*Aerobacter aerogenes*) in food vacuole. Note pale ('nuclear') areas in the bacteria are more concentrated towards the centre than corresponding areas in the mu particles.

Fig. 8. Mu particle with capsule, compared with mitochondria.

PLATE 3

Fig. 9*a*. Mu particles fixed with methanol+acetic acid, treated with tris buffer. Control for fig. 9*b*.

Fig. 9*b*. As fig. 9*a*, but treated with DNAase.

Fig. 10*a*. As figs. 9*a* and 9*b*, but fixed with osmic acid instead of methanol + acetic acid.

Fig. 10*b*. As fig. 10*a*, but treated with DNAase.

PLATE 4

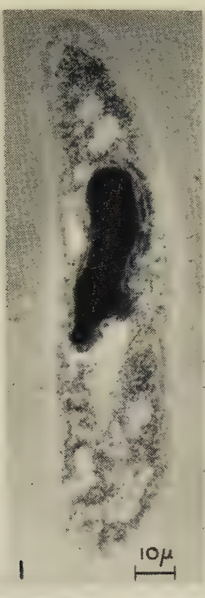
Fig. 11. Mu particle prepared by 'silver Feulgen' technique.

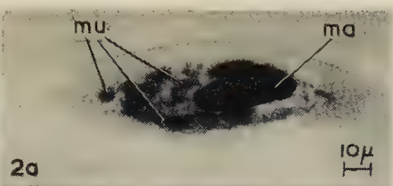
Fig. 12*a*. Mu particles fixed with osmic acid, hydrolysed with N-HCl and silvered. Control for fig. 12*b*.

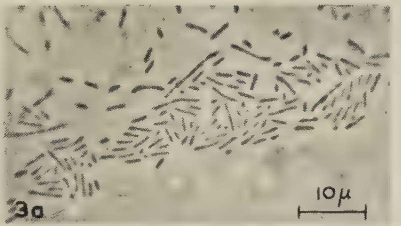
Fig. 12*b*. As fig. 12*a*, but treated with DNAase before silvering.

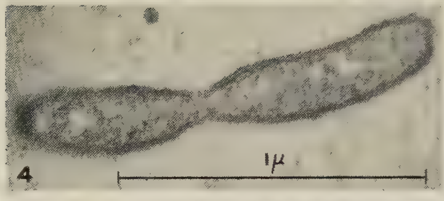
Fig. 13*a*. Mu particles fixed with methanol + acetic acid, rinsed in dilute buffered Ringer solution. Control for fig. 13*b*.

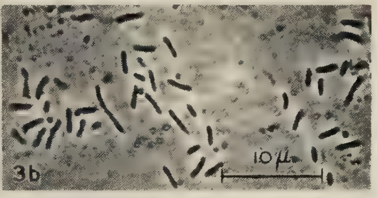
Fig. 13*b*. As fig. 13*a*, but treated with RNAase.

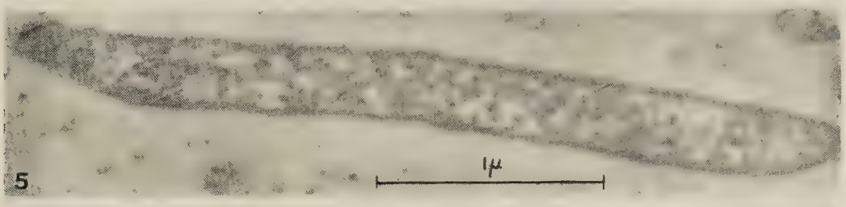


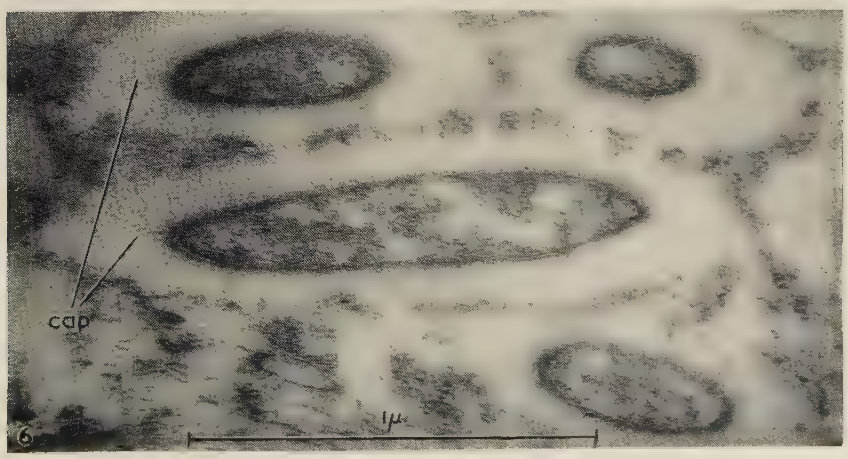












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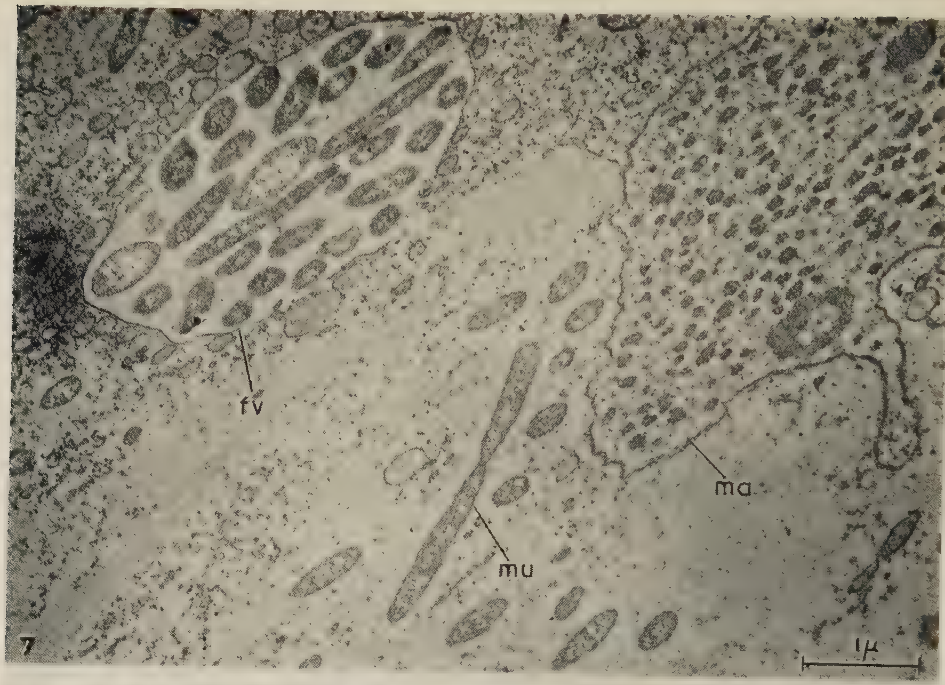

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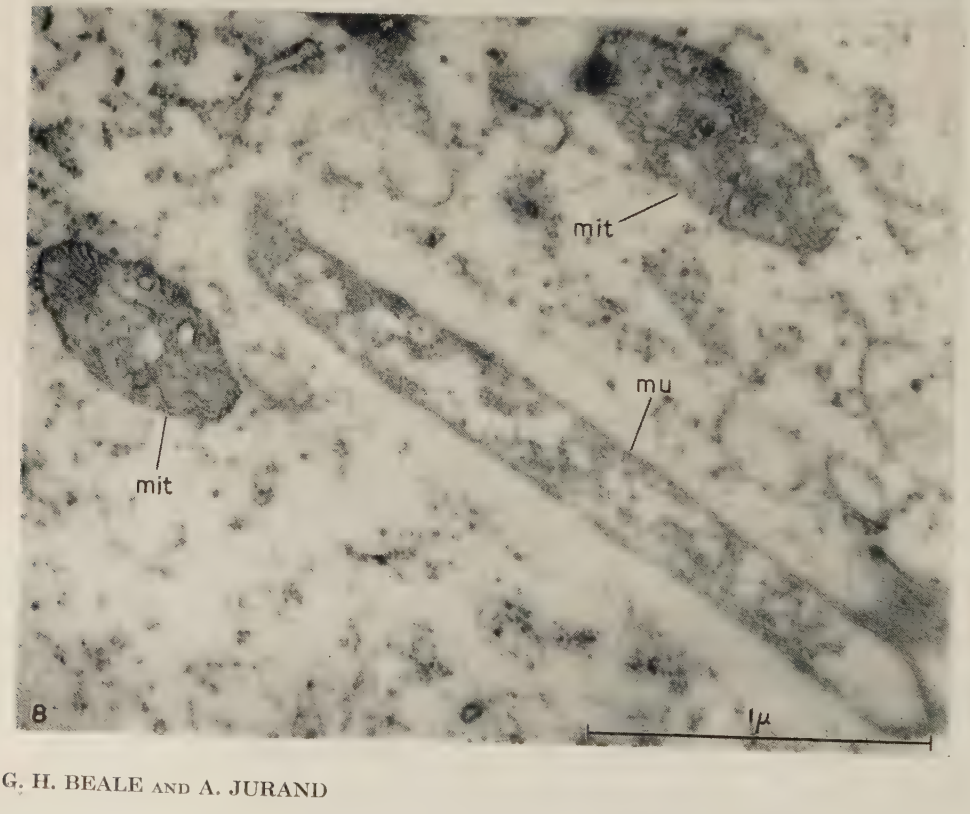
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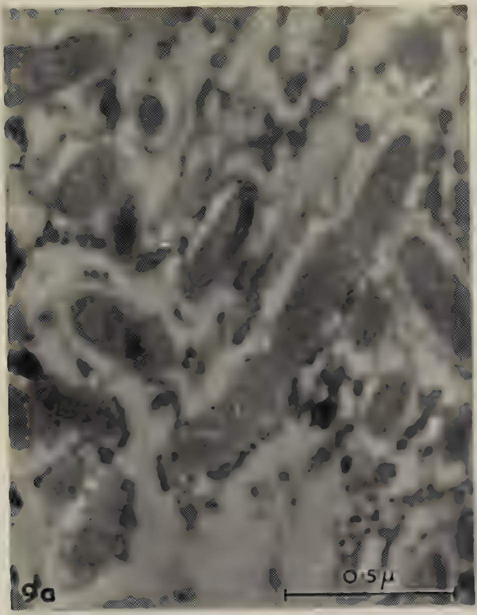

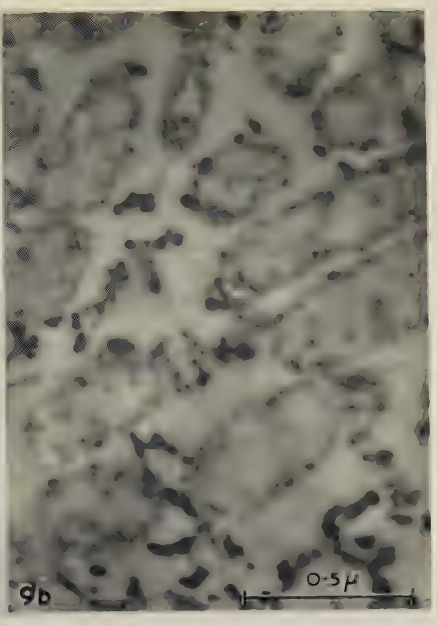

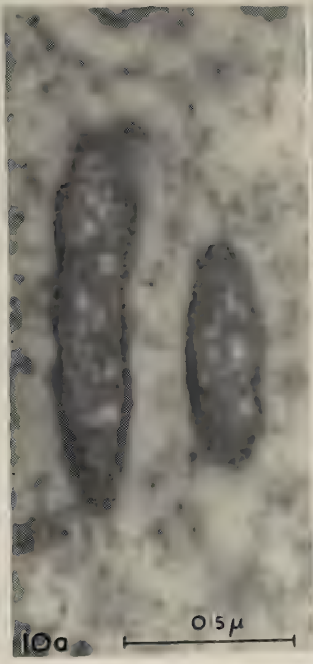

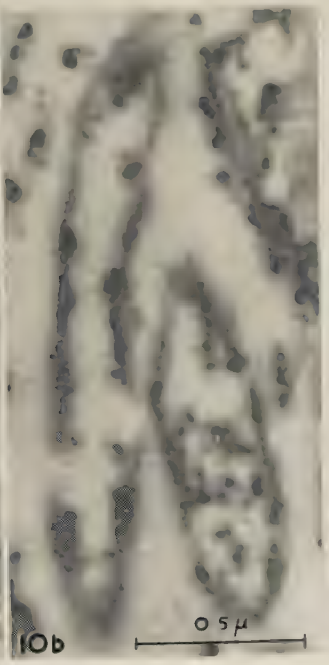

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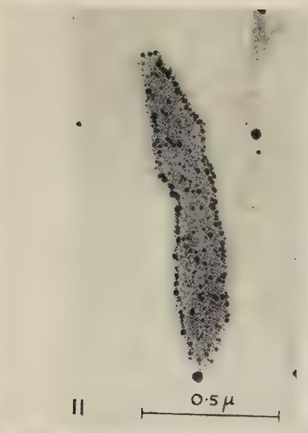
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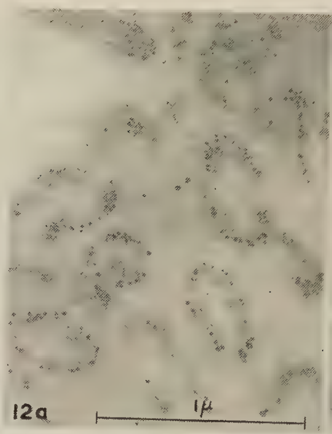
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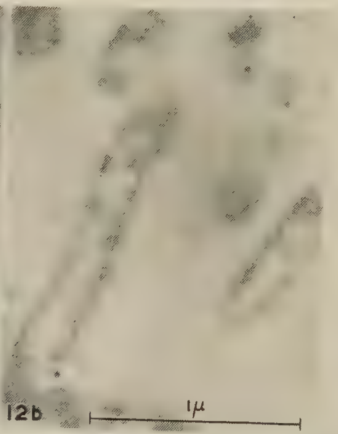
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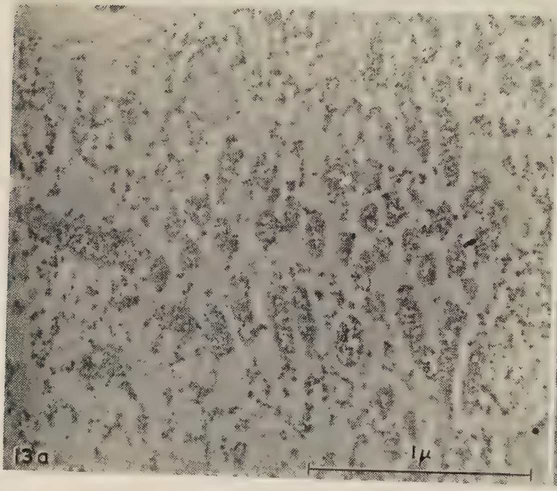
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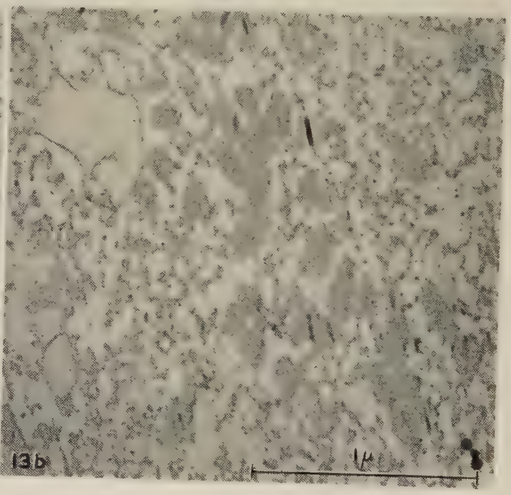
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Interference with Multiplication of Pc Phage by a Factor Produced by Exposure of *Pseudomonas aeruginosa* to Inactivated Pc Phage

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SUMMARY

Pseudomonas aeruginosa strain C10 when exposed to phage Pc which had been inactivated by heat or acid produced a factor which decreased the yield of Pc phage in a single cycle of growth on *P. aeruginosa* C10. Some of the factor appeared in the culture fluid, but most could be obtained only by disrupting the bacteria. The factor did not inactivate free Pc phage. It decreased phage yield most when it was mixed with *P. aeruginosa* C10 1 hr. before infection with Pc phage and was then kept present to the end of the experiment. When the factor was diluted out immediately after adsorption of Pc phage on *P. aeruginosa* C10, or when it was present only from the start of adsorption, it had less effect on phage yield.

INTRODUCTION

Isaacs & Lindenmann (1957) described a substance, which they named interferon, which was produced in tissue cultures by exposure of cells of the chick chorioallantoic membrane to previously inactivated influenza virus. They showed that in tissue culture the yield of influenza virus from the chick chorioallantoic membrane was decreased when interferon was added to the medium. Other authors (e.g. Henle, Henle, Deinhardt & Bergs, 1959; Ho & Enders, 1959) described similar substances produced by infecting cell cultures with other animal viruses. It was thought of interest to find whether similar activity could be obtained by exposing bacteria to inactivated phage. This work is reported here.

METHODS

Media. For preparation of phage stocks the chemically defined medium of Dickinson (1948) supplemented with 0·1 % (w/v) acid casein hydrolysate (vitamin free) was used. For all other work a papain digested meat broth was used.

Preparation of phage stocks. 1×10^8 infectious particles of Pc phage (Dickinson, 1954) and 5 ml. of a 24 hr. 37° culture of *Pseudomonas aeruginosa* C10 in nutrient broth were added to 100 ml. defined medium in a 500 ml. conical flask. The flask was incubated at 26° for 12 hr. on a rotary shaker. The host bacterium was then killed by heating at 55·5° for 30 min., and removed by centrifuging at 3600*g* for 6 hr. To ensure that no viable bacteria remained, the phage-containing supernatant fluid was then heated at 55·5° for a further 30 min.

Inactivation of Pc phage. Heat or acid was used for this; ultraviolet irradiation proved impractical for technical reasons. For each phage stock, small samples were taken and the minimum treatment required to decrease the titre to less than 10 infectious particles/ml. was determined. This treatment was then used for the bulk of the phage stock.

Production of phage-inhibitory factor. Fifty ml. of a 24 hr. broth culture of *Pseudomonas aeruginosa* C10 was added to 250 ml. of inactivated Pc phage suspension. After incubation at 37° for 1 hr. the bacteria were centrifuged, washed once to remove unadsorbed phage, and then suspended in 50 ml. broth and incubated at 37° for 2 hr. They were then centrifuged again and bacteria and culture fluid harvested separately. The culture fluid was heated at 60° for 30 min. to destroy remaining bacteria. The bacterial pellet was suspended in 5 ml. Ringer's solution and stored at 4° till the organisms were disrupted in a Hughes press cooled to $-20°$. After disruption (which was almost complete) the suspension of disrupted organisms was heated at 60° for 30 min., the bacterial debris removed by centrifuging, and the supernatant fluid harvested.

Tests for activity of phage-inhibitory factor. All tests were made by using single cycles of phage multiplication according to the method of Dickinson & Codd (1952). Phage yield (measured $2\frac{1}{2}$ hr. after the start of infection) when the phage-inhibitory factor was present throughout the experiment was compared with phage yield when it was absent throughout. After adsorption the phage was diluted to *c.* 400 particles/ml. Further dilutions were made just before counting, of 1/20 before the burst and 1/80 after it. This final dilution gave convenient numbers for counting (usually *c.* 200 plaques/plate for controls after the burst); it also diluted out any viable phage in the preparation being tested, and prevented any residual action of the phage-inhibitory factor.

RESULTS

Inactivation of Pc phage. The conditions required to inactivate the four phage stocks are shown in Table 1. The phage surviving after heat treatment was not inactivated after heating for 300 min. at 65°. The phage stocks treated by acid were shown still to contain viable Pc phage by incubating 5·0 ml. samples with *Pseudomonas aeruginosa* C10 overnight and then plating out for phage.

Table 1. *Conditions for inactivation of pseudomonad phage Pc*

	Treatment			Titre (plaques/ml.)	
Stock	Temp.	pH	Time (min.)	Initial	Final
				$(\times 10^{-9})$	
1	65°	7·2	202	9·6	4
2	67·5	7·2	210	4	2
3	20	3·0	30	1·5	< 1
4	20	2·5	13	5·9	< 1

Tests for phage-inhibitory activity. Culture fluids were tested undiluted, and disrupted cell preparations at a dilution of 1/10 to correspond to the volume of the culture fluid used. In a preliminary test no activity could be demonstrated with culture fluid when it was added at the time phage and host pseudomonad were first mixed, so for all other tests of activity culture fluid was added to the host organism 1 hr. before the phage was added. It was found (Table 2) that preparations from the four phage stocks had similar activities; the disrupted cell preparations were much more active than the culture fluids. Fluid and disrupted organisms from a culture of *Pseudomonas aeruginosa* C10 untreated with Pc phage were much less active; this culture fluid had no detectable effect, and the disrupted organisms decreased the phage yield only one-half compared with the decrease to one-seventh or less by preparations from organisms exposed to Pc phage.

Table 2. *Effect of preparations of phage-inhibitory factor on yield of pseudomonad phage Pc*

Preparation of inhibitor from stock	Yield of Pc phage as percentage of control: Culture fluid	Yield of Pc phage as percentage of control: Preparation of disrupted organisms
1*	81	16
2	72	2
3	64	Not tested
4	70	11
Uninfected culture	101	54

* In this preparation, activity was released into ten times the volume of culture medium used for the other preparations. Preparations were present from 1 hr. before the addition of Pc phage to *Pseudomonas aeruginosa* C10 to 2½ hr. afterwards. The yield at 2½ hr. was compared with that of Pc phage in complete absence of inhibitor preparation. All decreases in yield were significant ($P < 0{\cdot}02$ in Student's 't' test).

Mode of action of phage-inhibitory factor

A few experiments on the mode of action of the phage-inhibitory factor were made by using the disrupted-cell preparations from stock 4 at 1/10 dilution.

Effect on extracellular Pc phage. The phage-inhibitory factor caused no loss in titre of free Pc phage in broth (initial titre = $1{\cdot}7 \times 10^6$/ml.) during 2½ hr. at 37°.

Effect on multiplication of Pc phage. The effect of the phage-inhibitory factor when present throughout the experiment (i.e. from 1 hr. before the phage was added) was compared with the effect when it was present only to the end of adsorption. It was found (Table 3, Expt. 1) that a much bigger decrease in yield of phage was obtained when the phage-inhibitory factor was present throughout than when it was present only to the end of the adsorption period. This suggested that prolonged exposure to the phage-inhibitory factor was necessary for it to exert its maximum effect (either because of slow penetration into the host or change within the host to an active form). This suggestion was supported by comparing the effect of pre-exposing *Pseudomonas aeruginosa* C10 to the factor for 1 hr. before adsorption with the effect when it was present only from the beginning of adsorption (Table 3, Expt. 2). Unfortunately the inhibitor preparation was less active in this experiment than in previous ones, but it was clear that pre-incubation with the factor did increase its activity.

Other experiments

The phage-inhibitory factor was tested in a single experiment against Pa phage (Dickinson & Codd, 1952), a phage serologically unrelated to Pc phage but growing on the same host; the factor was active against phage Pa. The phage-inhibitory factor was also tested against Newcastle Disease virus in tissue culture by Dr Loïs Dickinson (personal communication) and was inactive.

Table 3. *The effect of phage-inhibitory factor on the multiplication of Pc phage on* Pseudomonas aeruginosa *C*10

All times were measured from the moment of mixing host bacterium and phage. The adsorption period was 10 min. Phage counts were made after dilution by the factors indicated.

Factor present		Phage titre	
From	To	10 min. (×1/20)	2½ hr. (×1/80)
		Experiment 1	
−1 hr.	+2½ hr.	14, 16, 15	33, 24, 25
−1 hr.	+10 min.	11, 14, 12	101, 58, 73
Absent		12, 11, 20	250, 248, 245
		Experiment 2	
−1 hr.	+2½ hr.	16, 15, 19	107, 133, 134
0 hr.	+2½ hr.	19, 19, 14	263, 220, 270
Absent		13, 9	250, 260, 288

DISCUSSION

The phage-inhibitory factor described here resembles interferon (Isaacs & Lindenmann, 1957; Lindenmann, Burke & Isaacs, 1957) in some ways: it is produced by the host cells in response to virus inactivated by very mild treatment; it does not inactivate free virus; it needs time to exert its full inhibitory effect; its activity is not restricted to the virus which caused its production. The phage-inhibitory factor differs from interferon in its greater stability to heat. It also is not liberated readily from the host bacteria, whereas interferon passes easily into the culture medium, but this may merely reflect the difference between the cell walls of bacteria and the chick chorioallantoic membrane.

We wish to thank Dr Loïs Dickinson for advice and criticisms during this work, and Drs M. Lumb and G. Woolfe for their interest and encouragement.

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With 1 *plate*
Printed in Great Britain

Replica Plating and Rapid Ascus Collection of Neurospora

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(*Received* 19 *March* 1960)

SUMMARY

Two colonial strains of *Neurospora crassa* have been found suitable for testing nutritional requirements by replica plating. One strain (*crisp*) has been replicated with a set of needles and the other (*crisp, ragged*) with filter paper. When ripe perithecia from *crisp* × *crisp* crosses are submerged in water, complete asci can be collected rapidly with a capillary pipette.

INTRODUCTION

The methods commonly used for collecting asci and determining nutritional requirements have limited the usefulness of *Neurospora crassa* for genetic investigations which require large numbers of complete asci. Neurospora asci have usually been separated from the contents of a perithecium that had been squeezed open on the surface of a block of agar. The asci often adhere to one another at their bases and they may be hard to separate. The growth of strains with wild-type morphology is so rapid that simultaneous determination of the nutritional requirements of many isolates by replica plating is not possible. The nutritional requirements of the progeny of such strains must be determined by transferring each isolate to test tubes containing appropriate media. Some improved methods have been reported in recent years. Strickland (1960) developed a rapid method for collecting large numbers of unordered asci which is based on the observation that individual asci are shot forcibly from the perithecia. Many of these asci can be recovered from an apposing surface as a cluster of eight spores. Reissig (private communication) applied a variation of the replica plating technique to Neurospora. Unfortunately, his method cannot be used to determine nutritional requirements. This paper is a description of methods for replication of two colonial strains of Neurospora and for collection of asci from homozygous crosses of one of these strains.

RESULTS

Neurospora strains with mutations crisp *and* ragged. When the two morphological mutations *crisp* (*cr, B 123*) and *ragged* (*rg, B 53*) (Perkins, 1959) are present in the same strain, growth is severely restricted. The colony formed has a diameter of only 2–3 mm. after 4 days' growth at 34°. When a dilute suspension of *crisp, ragged* conidia was spread on the surface of the agar in a Petri dish, the resulting colonies could be replicated by means of a continuous surface, such as velveteen or filter paper. The details of the replication procedure were essentially as described by Lederberg & Lederberg (1952). The replica plates, incubated at 34°, could be scored

3 days after replication (Pl. 1, fig. 1). Replication of *crisp, ragged* colonies arising from ultraviolet irradiated macroconidia was used to screen for nutritional mutants. A variety of nutritional, pigmented, and morphological differences can be scored in *crisp, ragged* strains. Crosses homozygous for both *crisp* and *ragged* produced very few ascospores and could not be used for recombination analysis.

Neurospora strain with crisp *mutation.* The single mutant strain *crisp, B123* (an allele of *crisp* with more restricted growth than the standard allele isolated by Lindegren), was also suitable for replication and crosses of *crisp* × *crisp* were fertile. Strains of *crisp* produce profusely conidiating colonies about 5 mm. in diameter after 2 days' growth at 34°. Colonies of *crisp* were replicated with needles so that the area of inoculation on the replica plates was small. This method of replication is similar to that developed by Forbes for *Aspergillus nidulans* (see Roberts, 1959). Asci are not shot forcibly from the perithecia of homozygous *crisp* crosses and they cannot, therefore, be collected by Strickland's method. However, large numbers of ordered asci were collected rapidly with the collection method (below) devised for these crosses. Moreover, ripe asci remained intact for a long time and they could be collected even after 3 months from cross-tubes which had been stored in the cold.

Collection of asci from homozygous crisp *crosses.* Asci could be collected beginning 3–4 weeks after inoculating the two parents together on to nutrient agar slopes. The top layer of agar, which contained embedded perithecia, was removed from the cross-tube and submerged in 10–15 ml. of sterile water in a Petri dish. When the cross was ripe, complete asci were extruded from the perithecia and settled to the bottom of the dish. The number of asci that were extruded could be increased by squeezing the perithecia. The asci were picked from the bottom of the dish and transferred to a block of 4 % (w/v) agar by means of a fine capillary pipette, suction being applied to the pipette by mouth through a rubber tube. The inside aperture of the capillary should not be much greater than the girth of an ascus. For greater efficiency, many asci are sucked up and blown out on to the centre of the agar block in one operation; these asci can then be picked up with the pipette, using additional water when necessary, and arranged so that the spores can be separated easily. The asci were routinely aged for 3–4 days at room temperature on the blocks to assure maximum germination; ageing may not be necessary when the crosses are older. When the spores were to be aged, the blocks were wetted with 5 % (w/v) sodium hypochlorite solution at least 10 min. before use.

The ascospores may be separated at any time during the ageing period. Separated spores were transferred to supplemented medium and heat-shocked immediately. In our experiments the spores were germinated on Petri plates so that they could be tested for nutrient requirements by replication without further preliminary manipulation.

Replication of crisp. The replicator that was used consisted of 32 nichrome needles mounted on a rectangular brass plate; two long screws acted as stops for orienting the plate over the needles (Pl. 1, fig. 2). The needles were sterilized with a Bunsen burner. Proper positioning of ascospores on the master plate was facilitated by using the heated needles of the replicator to sear visible depressions in the agar before the spores were transferred to the plate. Inoculated plates were then heat-shocked (with lids off) in a 60° oven for 1 hr. When much less than 25 ml. of 2·5 % (w/v) agar is used in the master plates, desiccation during heat shock may cause shrinkage and cracking, and the symmetry required for replication will be destroyed.

The plates were replicated 2–3 days after heat shock by inverting first the master plate and then the new plates on to the sterile needles. When more than one replica was desired, reseeding the replicator with the master was advisable before inoculating each new plate; reflaming the needles between successive replications from the same master plate was not necessary. The different types of testing media used in the replica plates were identified by adding Schilling food colouring to the medium. Contamination of the replica plates was negligible and *crisp* itself was never seen as a contaminant. All plates were incubated upside down to minimize contamination and condensation. The replica plates could be scored after 2 days' incubation at 34° (Pl. 1, fig. 3).

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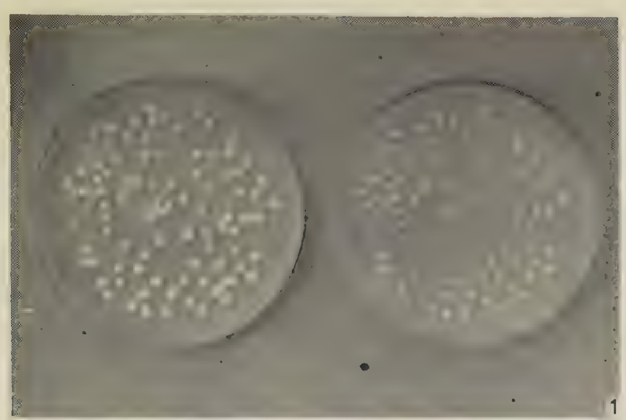
EXPLANATION OF PLATE

Fig. 1. Replication of *crisp*, *ragged*. The master plate is on the left and the replica on the right. (This plate was replicated with filter paper.)

Fig. 2. Replicator used with *crisp* (*B 123*).

Fig. 3. Replication of *crisp*. Each replica is labelled with the biochemical requirement it tests. The number below each colony on the master plate is the ascus and spore number (1–1 = ascus 1, spore 1). The two visible characters (col & al) do not show up clearly in the photograph.



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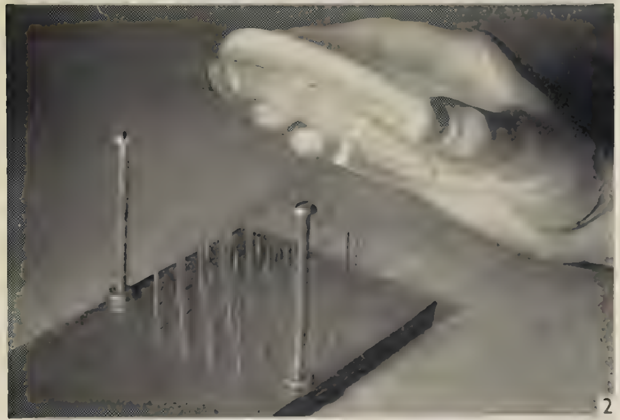

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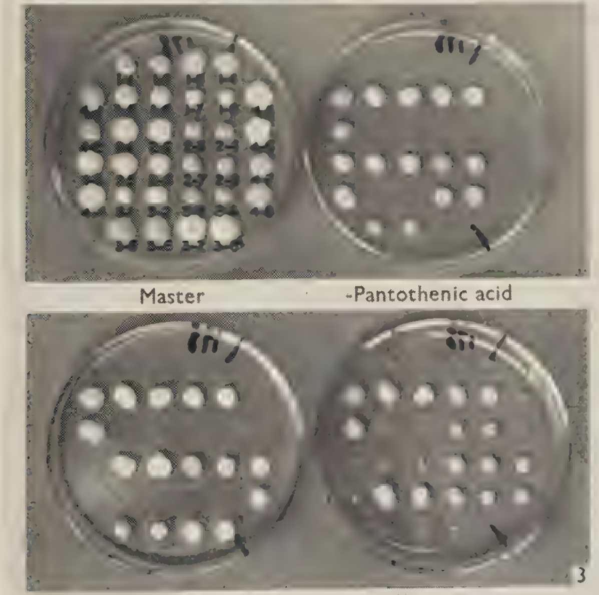

Master -Pantothenic acid

-Uridine -Thiamine

3

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A Technique for Obtaining Uniform Inocula of *Actinomyces israelii*

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(*Received* 24 *March* 1960)

SUMMARY

The anaerobic filamentous organism *Actinomyces israelii* grows as discrete colonies which sediment in static liquid media and are too large and variable in size to be suitable for quantitative inoculations of experimental media. A stirred-culture technique yielded uniformly small viable colonies of *Actinomyces israelii*. The microcolonies obtained still sedimented rapidly but could be kept in even suspension by bubbling nitrogen through the suspension. Such suspensions gave uniform inocula from a graduated Pasteur pipette as judged by total-nitrogen determinations and showed the growth characteristics of *A. israelii* grown in static liquid media.

INTRODUCTION

In pure culture the anaerobic filamentous organism *Actinomyces israelii* grows in liquid media as discrete coherent colonies of 0·5–2·5 mm. diameter; the supernatant medium remains clear at all stages of growth. The colonies, variously described as ‘cauliflower’ or ‘bread-crumb’ in appearance (Erikson, 1940; Erikson & Porteous, 1953), do not disperse on mild agitation of the culture vessel although the colonies of certain contaminated cultures will disintegrate (Erikson & Porteous, 1955). The colonies of different pure strains in liquid culture vary in consistency, from those which can be fragmented into smaller colonies by the tip of a Pasteur pipette to those which are so tough as virtually to resist fragmentation by this means. As a preliminary to a quantitative study of the nutritional requirements of *A. israelii* some means of obtaining a uniform inoculum suspension of this organism was required.

Howell & Pine (1956) studied thirteen strains of Actinomyces, five of which matched the partial description of *Actinomyces israelii* given above. These authors prepared their inocula by homogenizing a suspension of colonies in a tissue-grinder; the growth from such inocula was subsequently measured as the packed volume of harvested colonies, by determination of glucose utilization or, after repeatedly drawing and expelling the growing colonies through a sterile serological pipette at each time of measurement, by measuring the turbidity of the suspension. In the present work an attempt was made to repeat two of these homogenization techniques, but without success; colonies of all the strains used were too tough to submit to uniform fragmentation in an all-glass Potter & Elvehjem homogenizer or to pass

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into a serological pipette with consequent dispersion. Attempts to modify the latter method by using a sterile syringe and needle were also unsuccessful. The homogenizer technique is open to the objection that contamination during treatment of the culture may occur; Erikson & Porteous (1955) reported some of the difficulties consequent on slight contamination of *A. israelii* cultures. Attempts were also made to produce viable uniform suspensions of fragmented colonies by shaking with ballotini at +2° in a vibrating disintegrator (Mickle, 1948) for different periods (5–300 sec.), with distilled water, 1 % (w/v) NaCl, 0·01 M-phosphate buffer (pH 7) or the same buffer + 1 % (w/v) glucose, as suspending media. Shaking in the disintegrator for at least 30 sec. was required to produce an opalescent supernatant fluid, after allowing a further 2 min. for large fragments to sediment. Inocula taken from such supernatant fluids into medium S of Erikson & Porteous (1953) occasionally gave growth but no combination of the above conditions was found which consistently gave viable inocula of *A. israelii* by this technique. Eventually the technique described below was worked out; this proved suitable for the preparation of uniform viable inocula of *A. israelii*, and has been used for the past two years.

METHODS

Organisms. Of twelve strains of *Actinomyces israelii* available, one (strain 'Wills') was selected as showing particular resistance to fragmentation by the techniques mentioned above. Cultures were maintained as described by Erikson & Porteous (1953).

Culture media. In initial experiments, medium S and medium A of Erikson & Porteous (1953) were used in the first and second stages of the technique to be described. Subsequently chemically defined media (Howell & Pine, 1956) were used with equal success.

Sterilization. All apparatus was sterilized empty in the autoclave (15 lb./sq.in. for 30 min.) before addition of sterile medium.

Total-nitrogen determinations. Digestions were performed according to Chibnall, Rees & Williams (1943); the distillations were carried out in the Markham (1942) apparatus, the ammonia trapped in boric acid and determined as described by Conway & O'Malley (1942). Reagent blanks and standard $(NH_4)_2SO_4$ solutions were taken through the same procedure.

Apparatus. The stirred-culture apparatus used is shown in Fig. 1. The 500 ml. flasks contained magnetic stirrers and were plugged with cotton-wool and sterilized. The standard male joints, plugged with cotton-wool as indicated in Fig. 1, were separately sterilized with the tap-barrels removed. Sterile medium (100 ml.) added aseptically to each flask was inoculated from a stock culture of *Actinomyces israelii* by a Pasteur pipette. The cotton-wool plugs in the flasks were replaced aseptically by the sterile plugged male joints, each lightly smeared with sterile glycerol. Assembly of the apparatus was then completed with rubber-tubing joints as shown in Fig. 1 and the lubricated tap-barrels inserted. An indicator of anaerobiosis (Mackie & McCartney, 1949) was placed in tube *T*. The complete apparatus was evacuated through *E* at the water pump and filled with nitrogen three times.

Culture technique; first stage. The complete apparatus (Fig. 1) was incubated at 37° without stirring for the first 2 days after inoculation. Each flask was then stirred with a magnetic stirrer at 60 rev./min. for a period of 4 hr. during each of the succeeding 3 days; the stirring broke up the growing colonies.

Culture technique; second stage. At the end of the first stage of culture lasting 5 days the fine suspension of microcolonies was allowed to sediment in the culture flasks before transferring approximately equal parts of the sediment with a Pasteur pipette to 50 ml. volumes of fresh medium in each of several 100 ml. conical flasks. Sufficient inoculum was added to cover the bottom of the flask with a thin layer of colonies. The medium in the flasks was sealed with sterile liquid paraffin and incubated, without stirring, for 4–5 days at 37°. The colonies were then aseptically harvested and washed with sterile distilled water three times by decantation.

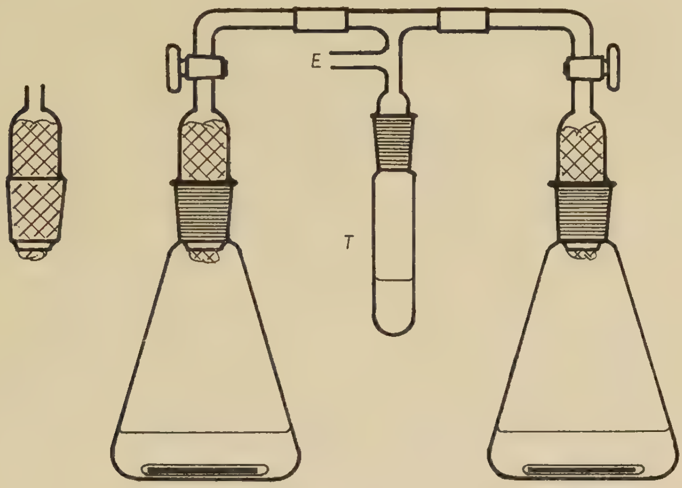

Fig. 1. The complete stirred-culture apparatus consisting of two 500 ml. conical flasks with standard female sockets; two plugged standard male cones fitted with stop-cocks; and a manifold connecting the flasks to a tube of anaerobic indicator and also providing an outlet *E* for filling the apparatus with nitrogen after evacuation.

Inoculation. The harvested and washed colonies were suspended in an equal volume of sterile distilled water in a test tube which was clamped at an angle of *c.* 20° above horizontal, over a micro-burner. A slow stream of nitrogen, sterilized by passage through sterile cotton-wool, was bubbled through the suspension of microcolonies to keep them evenly dispersed. A sterile calibrated dropping pipette was used to transfer *c.* 50 μl. of the suspension of colonies to each 12 × 75 mm. culture tube containing 2 ml. of experimental medium. At several intervals during inoculation of culture media a 50 μl. volume of the microcolony suspension was transferred to a sterilized digestion tube containing 2 ml. sterile water. Digestion of the contents of these tubes then permitted determination of the total-nitrogen content of the inocula.

RESULTS

The colonies obtained in the final suspension by the above technique were remarkably uniform in size. They were still sufficiently large (*c.* 0·2 mm. diam.) to sediment rapidly but could be kept in uniform suspension by gassing. Table 1 shows that the technique described provided uniform inocula. Contamination during inoculation was not experienced; inoculation rates as high as 200 tubes/hr. were accomplished.

The filamentous morphology of *Actinomyces israelii* (Erikson, 1940) precluded assessment of the viability of the inocula of microcolonies by comparison of viable with total counts. The colonies in the inoculum suspension were, however, microscopically typical; they grew to normal size in a single subculture and, in not more than four serial subcultures in static media, reverted to their normal nodular shape. Provided inocula were taken from similar phases of growth, into identical media, stirred-culture inocula showed no increase in lag phase as compared with unstirred-culture inocula of approximately the same size.

Table 1. *The total-nitrogen content* (μg. N) *of eight* 50 μl. *inocula of* Actinomyces israelii *obtained by the stirred-culture technique in three experiments*

Inoculum no.	Experiment (*a*)	(*b*)	(*c*)
	Total nitrogen (μg./50 μl.)		
1	21	17	20
2	21	17	22
3	20	19	20
4	20	20	22
5	22	19	23
6	21	19	22
7	20	18	21
8	18	19	21
Average	20	19	21
Range	(18–22)	(17–20)	(20–23)

DISCUSSION

The technique described, although undoubtedly tedious and time-consuming, has nevertheless yielded uniform inocula where other methods failed. The technique has the advantage that growth of the colonies takes place under conditions which prohibit entry of contaminants. The results suggest that the colonies produced by the technique were, apart from their size, identical with colonies grown in static media. The second-stage incubation was carried out for the following reasons: (i) it provided a useful indication of culture purity, the supernatant medium remaining characteristically clear and the colonies characteristically discrete; (ii) the smaller culture vessels facilitated harvesting of colonies; (iii) the amount of material available for experimental purposes was increased; (iv) it provided a period during which the microcolonies grew without becoming too large.

One of us (A.O.C.) was in receipt of a D.S.I.R. Research Studentship for the period October 1956 to October 1959.

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The Oxidation of Tricarboxylic Acid Cycle Intermediates by a Strain of *Corynebacterium erythrogenes*

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(*Received* 25 *March* 1960)

SUMMARY

Washed suspensions of *Corynebacterium erythrogenes* strain 142, grown on a yeast extract medium, were unable to oxidize some members of the tricarboxylic acid cycle immediately unless these compounds had been added to the growth medium. At 25° the organism eventually acquired the ability to oxidize these compounds; with succinate rapid oxidation was not obtained even after 6 hr. Rapid oxidation of succinate did occur when washed suspensions were incubated first with L- or D-malate. The lag periods were shortened by the addition of ammonium sulphate, but adaptation was prevented by chloramphenicol, 2:4-dinitrophenol, azide or an increase in temperature to 37°. Neither chloramphenicol nor the increase in temperature affected oxidation by adapted organisms; 2:4-dinitrophenol and azide were inhibitory, especially when added with the substrate. Extracts of unadapted organisms oxidized immediately all those compounds which intact organisms did not. Chloramphenicol and the respiratory uncoupling agents did not inhibit oxidations by the cell extracts. Extracts from adapted or unadapted bacteria reacted with succinate, malate and fumarate in the presence of hydroxylamine to form substances which gave coloured compounds with ferric chloride. The compounds with fumarate and malate were not identified. These compounds and succinhydroxamate were formed equally well by extracts of organisms grown in the absence or presence of the dicarboxylic acids. There was no evidence that the enzymes responsible for these reactions are directly concerned with the penetration of the acids into *C. erythrogenes*.

INTRODUCTION

Some micro-organisms either do not readily oxidize certain intermediates of the tricarboxylic acid (TCA) cycle (Krebs, Gurin & Eggleston, 1952; Moses, 1955; Davis, 1956) or will only do so after a lag period (Karlsson & Barker, 1948; Barrett, Larsen & Kallio, 1953; Kogut & Podoski, 1953). The accepted explanation of the phenomenon is that access of the added substrates to the enzymes is prevented by impermeability, which in some micro-organisms can be overcome by inducible transport processes (Kogut & Podoski, 1953; Clarke & Meadow, 1959). During an investigation of the metabolism of *Corynebacterium erythrogenes* lag periods before the oxidation of various tricarboxylic acid cycle intermediates were observed. The properties of intact organisms and of extracts have now been examined to see whether these results can similarly be ascribed to permeability barriers.

METHODS

Organism and growth of cultures

Corynebacterium erythrogenes strain 142 was originally isolated from cod skin by Dr J. M. Shewan (Department of Scientific and Industrial Research, Torry Research Station, Aberdeen). Its morphology and general growth characteristics resemble those of two other yellow-pigmented, porphyrin-excreting, coryneform organisms obtained from cod skin slime (Hodgkiss, Liston, Goodwin & Jamikorn, 1954). The organism was maintained by monthly subculture on to slopes of tryptic meat agar.

The basal growth medium used in the experiments to be described contained (g./l.): $Na_2HPO_4.12H_2O$, 16; KH_2PO_4, 1·3; NaCl, 8·5; $MgSO_4.7H_2O$, 0·12; yeast extract (Difco), 5·0; glucose, 18. The solution was made up without glucose, adjusted to pH 7·6 with N-NaOH and sterilized by autoclaving (10 min. at 115°). Glucose was then added as a separately autoclaved solution. Organic acids were added to this medium (containing glucose) as autoclaved solutions of their sodium salts, usually to a final concentration of 0·1 M.

Growth from a fresh 24 hr. tryptic meat agar slope culture was diluted in water to give organisms equivalent to *c.* 6 μg. dry wt./ml. This suspension (1·0 ml.) was used to inoculate 40 ml. basal medium contained in ⊥-shaped tubes (Monod, Cohen-Bazire & Cohn, 1951) of 120 ml. capacity. The tubes were incubated in a water bath at 25° and aerated by rocking at 36 oscillations/min. by a device similar to that of van Heyningen & Gladstone (1953).

Larger volumes of culture (up to 1 l.) were grown in 5 l. rotating flasks in an apparatus similar to that described by Mitchell (1949), in which the temperature could be controlled by having the flasks immersed in water at 25°.

All cultures were harvested towards the end of exponential growth (40 hr.) and washed twice with the culture volume of 0·073 M-NaCl.

Preparation of cell-free extracts

Ultrasonic preparations. Freshly harvested washed bacteria were resuspended in 0·05 M-phosphate buffer (pH 7·0) at a concentration equiv. 30–40 mg. dry wt. organism/ml. Volumes of about 15 ml. were subjected to ultrasonic vibration at 25 kc./sec. for 5 min. (Mullard Ultrasonic Generator, Type E 7590 B: Mullard Ltd, London). The transducer assembly was cooled by a stream of cold tap water. Unbroken organisms and coarse debris were removed by centrifuging at 25,000 *g* for 10 min. For some experiments the preparation was dialysed against 50 vol. of cold 0·05 M-phosphate buffer (pH 7·0) for 20 hr.

Fractions from lysozyme-treated organisms. Washed organisms (equiv. *c.* 1 g. dry wt.) were suspended in 144 ml. of a mixture consisting of 0·03 M-phosphate buffer (pH 7·0) + 0·04 M-NaCl + M-sucrose. To this lysozyme (Armour Laboratories Ltd, Hampden Park, Eastbourne, Sussex) was added to a final concentration of 200 μg./ml. The suspension was incubated at 25° for 3 hr. with occasional shaking by hand, and then centrifuged at 1500 *g* for 30 min. After washing in an equal volume of the above solution without lysozyme, the organisms were sedimented by centrifugation and lysed by resuspension in 100 ml. 0·05 M-phosphate buffer (pH 7·0). The lysate was mixed thoroughly by squirting through a pipette, and after about 10 min.

at room temperature was centrifuged at 25,000 *g* for 30 min. to yield a clear viscous supernatant fluid and a yellow pellet. The pellet was washed twice with a mixture of 0·07 M-NaCl + 0·05 M-phosphate buffer (pH 7·0) and finally suspended in 0·05 M-phosphate buffer (pH 7·0).

For some manometric experiments, lysozyme (0·1 ml. aqueous solution containing 2 mg./ml.) was added directly to suspensions of organisms in the presence or in the absence of M-sucrose in manometer vessels.

Estimations and reagents

Manometric methods. Oxygen uptake was measured in Warburg manometers with air as the gas phase. Enzyme preparation or washed suspension (equiv. *c.* 5–10 mg. dry wt. organism/2 ml. mixture of 0·07 M-NaCl + 0·05 M-phosphate buffer, pH 7·0) was placed in the main compartment. The substrate was in the side bulb and the centre well contained 0·2 ml. 20 % (w/v) KOH. Distilled water and any other additions were made to bring the total volume to 3 ml. Rates of O_2 uptake (Q_{O_2}) were expressed as μl./mg. dry wt. organism/hr.

Oxalacetic decarboxylase was measured by the method of Herbert (1955).

Hydroxamate formation. The incubation mixture contained: enzymic extract; tris buffer (2-amino-2-hydroxymethylpropane-1:3 diol), pH 7·4, 20 μmole; $MgCl_2$, 10 μmole; cysteine, 6 μmole; coenzyme A (CoA), 0·05 μmole; adenosine triphosphate (ATP), 10 μmole; hydroxylamine (hydroxylamine hydrochloride adjusted to pH 7·0 with KOH), 800 μmole; substrate, water to 2 ml.; in 1·5 × 11 cm. centrifuge tubes. After 2 hr. incubation at 30°, 1 ml. ferric chloride reagent (Berg, 1956) was added, and after centrifugation to remove precipitate the optical density at 540 mμ was measured in the Unicam SP. 600 spectrophotometer (Unicam Instruments Ltd, Cambridge), using cuvettes of 1 cm. light path.

Keto acids. Total keto acids were estimated by the method of Friedemann & Haugen (1943). For chromatographic identification the 2:4-dinitrophenyl-hydrazones were extracted into ethyl acetate and ascending chromatograms were developed in the solvent of Dagley, Fewster & Happold (1952) on Whatman no. 1 paper.

Protein. This was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumen (Armour Laboratories, Ltd, Hampden Park, Eastbourne, Sussex) as the standard.

Dry weight of washed suspensions. An EEL (Evans Electroselenium Ltd, Halstead, Essex) photoelectric colorimeter with a neutral density filter was used to assess the turbidity of washed suspensions. The instrument was calibrated by relating readings to the turbidities of washed suspensions of known dry wt. organism/ml.

Reagents. Analar grade or recrystallized chemicals were used whenever possible. Special materials were obtained from the following sources: coenzyme A (CoA, 70–75 % pure), triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) from the Sigma Chemical Co., St Louis, Mo., U.S.A.; chloramphenicol from Parke, Davis and Co., Hounslow, Middlesex.

RESULTS

Oxidation of intermediates by washed suspensions

Washed suspensions of *Corynebacterium erythrogenes* harvested after growth for 40 hr. in the basal medium were tested for their ability to oxidize members of the

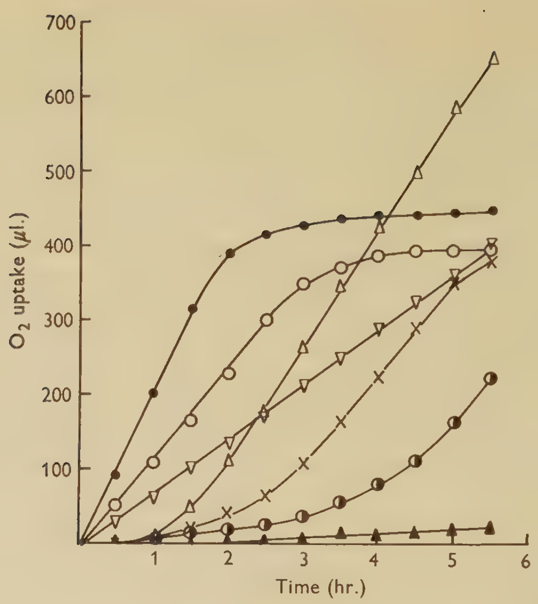

Fig. 1. Oxidative ability of *Corynebacterium erythrogenes* grown on the basal medium alone. Substrates (10 µmole), ▲—▲, succinate; ◑—◑, fumarate; ×—×, L-malate; ▽—▽, α-oxoglutarate; △—△, citrate; ○—○, acetate; ●—●, pyruvate. Temperature 25°. Endogenous O_2 uptake has been subtracted.

tricarboxylic acid cycle and related compounds. With acetate, pyruvate and α-oxoglutarate, the rates of O_2 uptake were linear from the time of substrate addition; with citrate, malate and fumarate, there was a lag before rapid rates of oxidation were obtained; with succinate there was only a slight uptake of oxygen even after 6 hr. (Fig. 1). The lag period with a particular intermediate could be abolished by incorporating it in the growth medium, and the patterns of oxidation obtained under these conditions are shown in Table 1.

Table 1. *Oxidative ability of washed suspensions of* Corynebacterium erythrogenes *grown on the basal medium with various supplements*

The values have been corrected for the oxygen uptakes in the absence of substrate. Temperature 25°.

	Substrates added to the basal growth medium						
	None	Acetate	Succinate	Malate	Fumarate	Citrate	α-Oxo-glutarate
	Q_{O_2} values						
Substrate							
Pyruvate	30	—	47	28	25	—	48
Acetate	16·6	31·5	—	—	—	—	—
Succinate	(*)	(*)	19·5	35	28	(*)	(*)
L-Malate	(18·8)	(19)	17·2	29	26	(*)	(*)
Fumarate	(18)	(18·4)	17	30	25	(*)	(*)
Citrate	24	19	30	24	13	61	(18)
α-Oxoglutarate	11	19·2	7·8	3·2	5·3	42	43·5
None	5·8	7·4	6	18	6·4	8·7	6·4

( ) Indicates lag period; (*) indicates the linear rate was not achieved after 5 hr.; — indicates not teste

Factors affecting the lag period

The inability of organisms grown on the basal medium to oxidize malate, fumarate, succinate and citrate without a lag suggested that induced enzyme formation was required before these substrates could be metabolized. The effect of various treatments known to affect induced enzyme formation was therefore investigated.

Chloramphenicol. Chloramphenicol, 20 μg./ml., completely prevented the increase in the rates of oxidation normally shown by organisms grown in the basal medium; the oxidation of compounds by bacteria grown in their presence was unaffected.

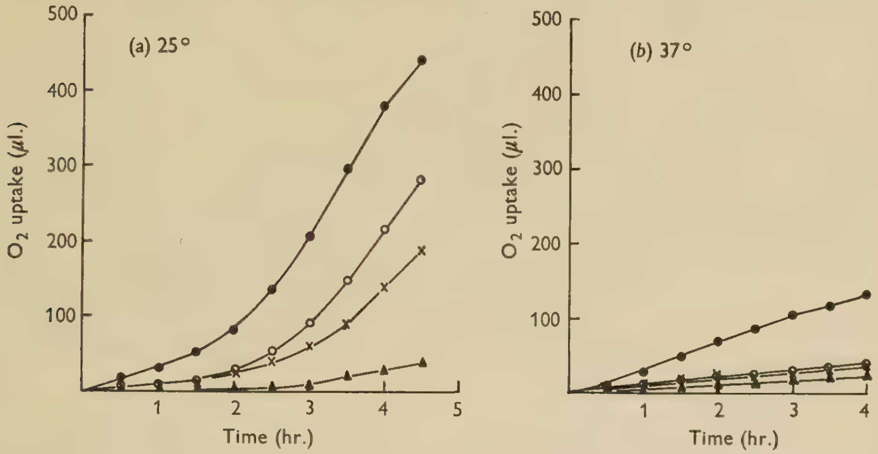

Fig. 2*a*, *b*. Effect of temperature on the adaptation of washed suspensions of *Corynebacterium erythrogenes* to tricarboxylic acid cycle intermediates. Substrates (10 μmole), ●—●, citrate; ○—○, L-malate; ×—×, fumarate; ▲—▲, succinate. Temperature (*a*) 25° and (*b*) 37°. Endogenous O_2 uptakes have been subtracted.

Temperature. The growth of *Corynebacterium erythrogenes* is inhibited at temperatures greater than 25°; the effect of higher temperatures on the adaptive response was therefore examined. At 37°, malate, fumarate, succinate and citrate were oxidized by organisms grown on the basal medium alone, but the rates of oxidation were slow and did not show the increase with time which was characteristic of suspensions incubated at 25° (Fig. 2). The increase in temperature was not directly inhibitory to oxidation; for example, in one experiment with organisms grown in the presence of malate the Q_{O_2} for malate was increased from 25 to 51 on raising the temperature from 25° to 37°.

2:4-*Dinitrophenol and sodium azide.* The adaptive response was completely abolished by 2:4-dinitrophenol (DNP; at 0·00036 M) and azide (0·0036 M). These inhibitors also interfered with the utilization of substrates by adapted organisms (Table 2), and higher concentrations of DNP (0·0018 M) suppressed malate oxidation entirely. This effect of DNP and azide on adapted organisms was most marked when substrate and inhibitor were added together; much less slowing of the rate of oxidation was produced if the inhibitor was added some time after the substrate.

Added nitrogen and energy sources. The long time-lag before washed suspensions began to oxidize malate and fumarate at a rapid rate suggested that some factor

Table 2. *Effect of* 2:4-*dinitrophenol and azide on the rates of oxidation by fumarate-grown* Corynebacterium erythrogenes

Standard manometric conditions with substrates (10 μmole) added from side arm. Temperature 25°.

	No inhibitor	Azide (0·0036 M)	2:4-Dinitrophenol (0·00036 M)
	O_2 uptake (μl./hr.)		
Substrate			
None	43	46	72
Succinate	213	106	122
L-Malate	183	96	92
Fumarate	188	86	107

connected with protein synthesis might be limiting. The large internal store of amino acids which Gram-positive micro-organisms contain is usually sufficient for induced enzyme formation, though Spiegelman & Dunn (1947) found that the process may be speeded in the presence of an external source of nitrogenous compounds. With *Corynebacterium erythrogenes* the addition of 100 μmole ammonium sulphate to washed suspensions under the standard manometric conditions lessened the time required for the achievement of linear rates of oxidation of malate and fumarate, though not of citrate. With succinate there was a slight increase in the rate of oxidation after about 6 hr.

The availability of energy may have a marked effect on the kinetics of induced enzyme synthesis (see Monod, Pappenheimer & Cohen-Bazire, 1952), and Clarke & Meadow (1959) found that the formation of the citrate transport mechanism in *Pseudomonas aeruginosa* was more rapid when a small amount of fumarate was added so that the organisms were not solely dependent on citrate for energy supply. The addition of a number of readily oxidizable substrates to washed suspensions of *Corynebacterium erythrogenes* did not diminish the lag periods with citrate, malate, fumarate or succinate. Glucose, which has an inhibitory action on the formation of some induced enzymes (Neidhardt & Magasanik, 1957), including the transport mechanism required for citrate utilization by *Aerobacter aerogenes* (Davis, 1956), had no effect on the adaptation processes of *C. erythrogenes*.

The effect of adaptation to other substrates. Growth in the presence of L-malate or fumarate or succinate yielded organisms capable of oxidizing all three substances without a lag (Table 1). The possibility was examined that the long time lag shown towards succinate by washed suspensions which had been grown in the basal medium alone might be influenced by the adaptation of the harvested organisms to malate or fumarate. Bacteria were adapted to a small amount of L-malate (2·5 μmole) in manometer vessels and after 4 hr., when the O_2 uptake with this substrate had ceased, 10 μmole succinate were added, with or without chloramphenicol, from the second side arm (Fig. 3). The immediate rapid oxidation of succinate which occurred was unaffected by the presence of chloramphenicol, and is to be contrasted with the slow rate of O_2 uptake in the control after incubation for 6 hr. with succinate. In other experiments it was found that incubation of washed suspensions with D-malate for 3 hr. enabled the immediate rapid oxidation of L-malate, fumarate and succinate.

Oxidation of intermediates by cell-free extracts

If, as with other organisms, the inability of *Corynebacterium erythrogenes* to oxidize TCA cycle intermediates rapidly is due to a permeability barrier which is overcome during the period of adaptation, cell-free preparations should have a wider range of oxidative activity than intact organisms.

Ultrasonic preparations. Malate, fumarate and citrate were all oxidized rapidly from the time of addition by ultrasonic preparations of organisms grown on the basal medium alone (Table 3). Disruption of the bacteria, however, caused a loss of the ability to oxidize α-oxoglutarate, and attempts to demonstrate α-oxoglutarate dehydrogenase in these ultrasonic preparations by the method of Kaufman (1955) were unsuccessful. Succinate was oxidized only slowly by ultrasonic preparations, though definite succinic dehydrogenase activity was shown by using phenazine methosulphate as hydrogen acceptor (Singer & Kearney, 1957).

Table 3. *Oxidation of some tricarboxylic acid cycle intermediates by ultrasonic extracts of* Corynebacterium erythrogenes *grown on the basal medium alone*

Manometer vessels contained in 3 ml. final volume: enzymic preparation, 22 mg. protein; phosphate buffer (pH 7·0) 50 μmole; TPN, 0·125 μmole; DPN, 0·14 μmole; $MgCl_2$, 2 μmole; substrate, 20 μmole (added from side arm); 0·2 ml. 20 % KOH (centre well). Temperature 25°.

Substrate	O_2 uptake during initial 30 min. (μl.)
None	15
Acetate	20
Citrate	220
Fumarate	210
L-Malate	190
α-Oxoglutarate	15
Pyruvate	50
Succinate	30

Lysozyme-treated organisms. During an investigation into various methods of disrupting the organism it was found that its potential oxidative ability when grown on the basal medium alone could be shown by treatment with lysozyme. Organisms treated with lysozyme in the absence of sucrose oxidized malate and fumarate at an initial rate which was the same as that of equivalent suspensions of intact bacteria grown in the presence of these compounds; however, after the consumption of about 1 atom O_2/mole substrate the rate became slower. Citrate was oxidized at the latter rate from the time of tipping, though faster oxidation could be obtained by the addition of DPN (0·14 μmole) and TPN (0·125 μmole). The lyzozyme-treated suspensions oxidized succinate and α-oxoglutarate very slowly. Examination of the contents of the manometer vessels at the end of the experiment revealed pyruvate and oxaloacetate as the products of the oxidation of both malate and fumarate, and the formation of α-oxoglutarate from citrate.

Organisms treated with lysozyme in the presence of M-sucrose showed an adaptive type of response with malate, fumarate and citrate. As with intact organisms the adaptation was prevented by chloramphenicol, which had no effect on the oxidation of substrates by bacteria treated with lysozyme in the absence of sucrose (Fig. 4).

On centrifugation at 25,000 *g* for 30 min., suspensions of *Corynebacterium erythrogenes* which had been treated with lysozyme in the presence of M-sucrose and subsequently lysed separated into a clear viscous supernatant fluid and a yellow pellet. The pellet was washed twice in a mixture of 0·07 M-NaCl + 0·05 M-phosphate buffer (pH 7·0) and finally suspended in 0·05 M-phosphate buffer (pH 7·0). The supernatant fluid and pellet fractions were tested for various enzymic activities.

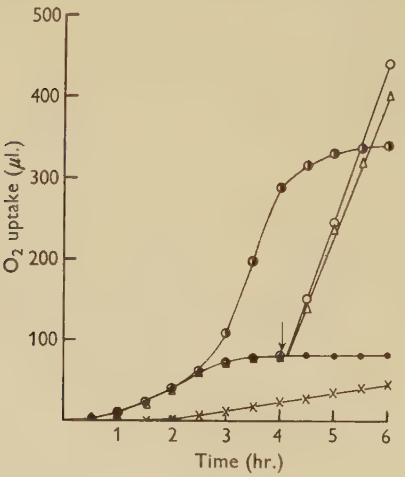

Fig. 3

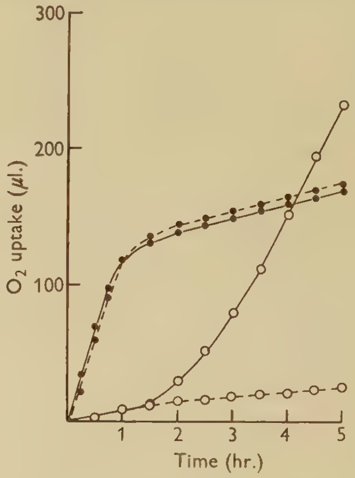

Fig. 4

Fig. 3. Effect of adaptation of washed suspensions of *Corynebacterium erythrogenes* to malate on the rate of oxidation of succinate. Substrates, ◑—◑, L-malate (10 µmole); ●—●, L-malate (2·5 µmole); ×—×, succinate (10 µmole) added at zero time. Addition of succinate (10 µmole), with, ○—○ or without, △—△, chloramphenicol (20 µg/ml.) at point shown by arrow after the completion of oxidation of 2·5 µmole L-malate. Temperature 25°. Endogenous O_2 uptakes have been subtracted.

Fig. 4. Effect of chloramphenicol and the oxidation of fumarate by suspensions of *Corynebacterium erythrogenes* treated with lysozyme in the presence or absence of sucrose. Standard manometric conditions with lysozyme (200 µg.) in each vessel and fumarate (10 µmole) added from side arm. Sucrose (M) present, ○; absent, ●. Chloramphenicol (20 µg./ml.) present, - - -; absent, ——. Temperature 25°. Endogenous O_2 uptakes have been subtracted.

The pellet contained all the malate oxidizing activity of the whole lysate, while all the oxaloacetic decarboxylase was located in the supernatant fluid. A similar distribution of malic dehydrogenase was indicated by using ferricyanide as hydrogen acceptor (Cohn, 1956), showing that localization of the cytochrome system in the pellet was not responsible for these results. Comparison of the two fractions obtained from organisms grown in the basal medium alone or with added fumarate revealed no marked difference in the activities, in terms of protein content, of malic dehydrogenase and oxalacetic decarboxylase. The pellet fraction alone was unable to oxidize fumarate as fast as the whole lysate. However, this rate could be attained by adding the supernatant fluid, showing that fumarase was present in both fractions.

The distribution of cytochromes between the supernatant fluid and the pellet fractions was determined by shaking portions of each with a small amount of sodium dithionite followed by examination with the hand spectroscope. There was strong absorption at 560 mμ with the pellet, indicating the presence of cytochrome *b*, but no absorption bands were detected in the supernatant fluid.

The effect of 2:4-*dinitrophenol and azide*

2:4-Dinitrophenol or azide not only prevented the adaptation to various intermediates of the TCA cycle but also slowed their oxidation by whole adapted organisms (Table 2). A number of results indicated that the latter effect was not due to direct interference with respiration but with the mechanism for the transport of the substrates across the permeability barrier. Thus concentrations of 2:4-dinitrophenol (0·00036 M) or azide (0·0036 M) which decreased the Q_{O_2} value of L-malate, fumarate and succinate to 30–35 % of the control values produced no inhibition of the oxidation of other compounds such as acetate and glucose. There was also very little effect of 2:4-dinitrophenol or azide on the initial rate of oxidation of L-malate and fumarate by cell-free extracts, prepared either by lysozyme treatment or by ultrasonic disintegration (Fig. 5).

Attempts to show activation of substrates

Both 2:4-dinitrophenol and azide are well known as uncouplers of oxidative phosphorylation and they may have a common point of attack on some endergonic process which is required for the mechanism of penetration. Since the energy-requiring process might be an activation of the substrate such as that proposed by Mitchell (1957) and Mitchell & Moyle (1959) an attempt was made to detect the formation of reactive compounds by cell-free preparations by using hydroxylamine as trapping agent. Initially, ultrasonically-disrupted preparations of *Corynebacterium erythrogenes* which have been grown in the presence of succinate were used, with the dicarboxylic acids and acetate as substrates.

Dialysed extracts of ultrasonically disrupted bacteria which had been grown in the presence of succinate were incubated with cysteine, ATP, CoA, $MgCl_2$, hydroxylamine and substrate for 2 hr. at 30°. On the addition of ferric chloride reagent, coloured compounds were formed with each of the substrates, malate, fumarate, succinate and acetate. Deproteinization of the incubation mixture, followed by ethanol extraction and chromatography according to the method of Stadtman & Barker (1950), showed that the compounds formed with acetate and succinate were the corresponding hydroxamates. The same method of extraction and chromatography applied to the incubation mixtures containing malate and fumarate failed to reveal compounds reacting as hydroxamates. In spite of this, the reaction of enzymic extracts with malate and fumarate was possibly related to the process of penetration, and further investigation was therefore made.

The effect of cofactors. The incubation conditions were as before. With fumarate the amount of coloured compound formed was independent of the presence of added CoA, ATP, cysteine and divalent metal ion. With L-malate, however, maximum colour development was obtained only in the complete system; omission of ATP or CoA and cysteine resulted in approximately 50 % less absorption at 540 mμ (Table 4). Magnesium or manganese ions were also needed for maximum activity with malate. Since both CoA and ATP were necessary for the formation of succinhydroxamate it is possible that the coloured malate compound is produced by two mechanisms, one of which requires CoA, ATP and divalent metal, and another which is independent of these compounds. The comparison of extracts from unadapted organisms with those from organisms grown in the presence of malate or succinate revealed no marked difference in activities.

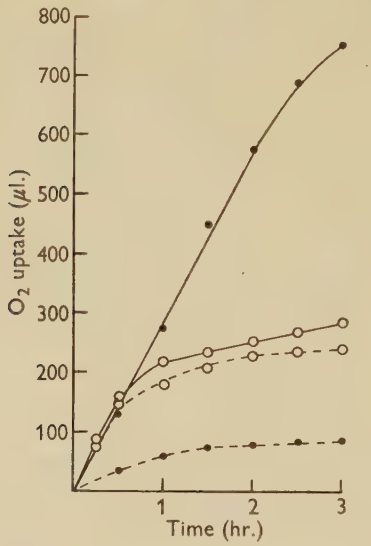

Fig. 5

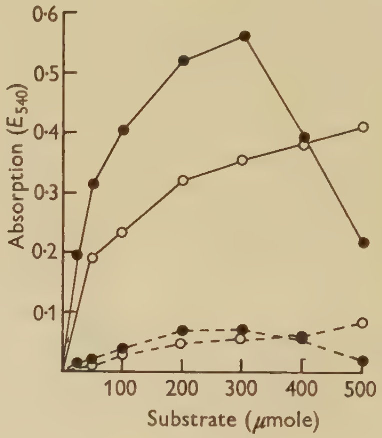

Fig. 6

Fig. 5. Effect of azide on the oxidation of malate by malate-grown *Corynebacterium erythrogenes*. Standard manometric conditions with L-malate (20 µmole) added from side arm. Lysozyme (200 µg.) present, ○; absent, ●. Sodium azide (0·0036 M) present, – – –; absent, ——. Temperature 25°. Corresponding endogenous O_2 uptakes have been subtracted.

Fig. 6. Effect of substrate concentration on the reaction of dialysed, ultrasonic extracts of *Corynebacterium erythrogenes*, grown on basal medium alone, with hydroxylamine and L-malate or fumarate. Experimental conditions as in Table 4. Substrates: ○, L-malate; ●, fumarate. Boiled extracts, – – –. Values have been corrected for the extinction at 540 mµ in the absence of substrate.

The effect of fluoride. The formation of acyl hydroxamates in the absence of CoA and ATP was reported by Lipmann & Tuttle (1950) and by Elliott (1956) and appears to be due to a non-specific reaction catalysed by lipase, especially when high concentrations of substrate are used. Potassium fluoride, which has been used to prevent this action of lipase (Lipmann & Tuttle, 1950; Elliott, 1956), had no effect on the reaction of *Corynebacterium erythrogenes* ultrasonic preparations with malate and fumarate, either in the presence or the absence of CoA, ATP and cysteine.

The effect of concentration of extract, time of incubation and substrate concentration. The amounts of coloured compounds formed with malate and fumarate were proportional to the amount of ultrasonic preparation present (Table 5) and to the incubation times up to 2 hr. Colour produced in the reaction increased with increasing substrate concentration, though high concentrations of fumarate were inhibitory (Fig. 6). While the reaction of the preparations with fumarate and malate

Table 4. *The reaction of ultrasonic preparations of* Corynebacterium erythrogenes *with dicarboxylic acids in the presence of hydroxylamine*

The complete incubation mixture contained: tris-HCl buffer (pH 7·4) 20 μmole; $MgCl_2$, 10 μmole; cysteine, 6 μmole: ATP, 10 μmole; CoA, 0·05 μmole; hydroxylamine, 800 μmole; substrate, 300 μmole; dialysed ultrasonic preparation, equiv. 3–5 mg. protein; water to 2 ml. Incubation time 2 hr. at 30°. Absorption measured in 1 cm. cuvettes after adding 1 ml. $FeCl_3$ reagent and centrifuging.

Source of ultrasonic preparation	Substrate	Omissions from the complete mixture: None	CoA + cysteine	ATP	CoA + cysteine + ATP
		Light absorption ($E_{540} \times 10^{-2}$/mg. protein)			
Unadapted organisms	None	2·3	1·5	1·5	0·5
	L-Malate	9·4	5·1	6·0	4·7
	Fumarate	24·3	24·3	26·4	25·0
	Succinate	15·2	2·4	1·9	1·2
Succinate-grown organisms	None	2·1	1·0	2·0	1·2
	L-Malate	11·9	6·3	7·1	6·0
	Fumarate	36·8	35·7	37·7	37·7
	Succinate	15·6	3·3	2·8	1·7
Fumarate-grown organisms	None	1·8	1·3	1·0	0·5
	L-Malate	8·5	5·5	5·6	4·0

appeared to be enzymic, it was possible that the coloured compounds were not formed from these substances directly but from products of their further metabolism. Under the conditions of these experiments, namely incubation in open centrifuge tubes, malate and fumarate with all the additions except hydroxylamine gave rise to keto acids which were detected as the 2:4-dinitrophenylhydrazones. However, several observations made it unlikely that keto acid formation could account for the results. Thus no coloured compounds were formed on the addition

Table 5. *Effect of protein concentration on the reaction of ultrasonic preparations of* Corynebacterium erythrogenes *with fumarate and hydroxylamine*

Experimental details as in Table 4. Incubation time 2 hr. at 30°. The values of controls in the absence of substrate have been subtracted.

Extract (mg. protein)	Unheated preparation	Heated preparation*
	Light absorption ($E_{540} \times 10^{-2}$/mg. protein)	
None	0·10	0·10
0·54	0·18	0·09
1·07	0·25	0·09
2·14	0·37	—
3·20	0·47	0·06
4·28	0·57	—
5·35	0·61	0·06

* Enzymic activity of preparation destroyed by heating in water bath at 100° for 10 min.

of ferric chloride reagent to the incubation mixture unless hydroxylamine was present, and anaerobic incubation greatly diminished the amount of keto compounds formed without significantly affecting the reaction between malate and hydroxylamine (Table 6). Also, dialysis of the ultrasonic preparation against water for 16 hr. instead of against 0·05 M-phosphate buffer (pH 7·0) inactivated the mechanism responsible for the formation of the unknown compound but did not diminish keto acid formation.

Table 6. *Effect of aerobic and anaerobic incubation of ultrasonic preparations of* Corynebacterium erythrogenes *on* (*a*) *the reaction with malate and hydroxylamine,* (*b*) *the formation of keto acids from* L-*malate*

Aerobic incubation in 11 × 1·5 cm. centrifuge tubes. Anaerobic incubation in Thunberg tubes; gas phase N_2; L-malate added from side arm. Incubation time 2 hr. at 30°. (*a*) Experimental details as in Table 4. Results expressed as $E_{540} \times 10^{-2}$/mg. protein/2 hr. (*b*) Experimental details as in Table 4 but hydroxylamine omitted. 2:4-Dinitrophenylhydrazones were prepared and amounts formed expressed as E_{440}/mg. protein/2 hr.

		Omissions from the complete incubation mixture							
		None				CoA + ATP + cysteine			
		Aerobic		Anaerobic		Aerobic		Anaerobic	
Source of extract	Substrate	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Fumarate-grown organisms	None	2·33	0	1·96	0	1·28	0	1·14	0
	L-Malate	9·0	1·34	9·5	0·26	3·98	1·37	4·26	0·19
Fumarate-grown organisms (ultrasonic preparation dialysed against water)	None	1·6	0	—	—	—	—	—	—
	L-Malate	2·45	1·3	—	—	—	—	—	—

Localization of the enzymes responsible for the reactions of malate, fumarate and succinate with hydroxylamine

If the enzymes responsible for the activation of succinate and for the formation of the coloured compounds with fumarate and L-malate are concerned with the penetration of these substances into the bacteria, a differential distribution of them between cytoplasm and cell membrane might be anticipated. High-speed centrifugation (105,000 *g*) of the ultrasonic preparations caused the sedimentation of a dark red pellet, probably the small particle fraction corresponding to fragmented cell membrane (see Mitchell, 1959). The supernatant fluid from such centrifugation was yellow and, since it had malic dehydrogenase activity, it still contained material derived from the cell membrane. Nevertheless, if activating enzymes are situated in the membrane there might be relatively different amounts present in the two fractions. Using the same conditions as in Table 4 the supernatant fluid and pellet fractions were tested for their ability to form succinhydroxamate and the reactive compounds from malate and fumarate. The results showed that the supernatant fluid had greater activity per unit weight of protein than the pellet fraction. A similar experiment was carried out with the pellet and supernatant fluid fractions from organisms lysed with lysozyme. The supernatant fluid not only had greater enzymic activity than the pellet per unit weight of protein (Table 7) but also, since it contained more protein, greater total activity.

Table 7. *Reaction of supernatant fluid and pellet fractions from lysed* Corynebacterium erythrogenes *with dicarboxylic acids and hydroxylamine*

Experimental conditions as in Table 4. Incubation time 2 hr. at 30°.

Organism	Substrate	Pellet	Supernatant fluid
		Light absorption ($E_{540}\times 10^{-2}$/mg. protein)	
Unadapted	None	0·9	1·0
	L-Malate	2·3	7·3
	Fumarate*	2·6	11·2
	Succinate	0·9	8·8
Malate-grown	None	1·7	1·6
	L-Malate	2·4	10·2
	Fumarate*	2·6	13·0
	Succinate	2·7	6·3

* CoA + cysteine + ATP were omitted from the tubes containing fumarate.

DISCUSSION

The adaptation to oxidize various TCA cycle intermediates which has been described for *Corynebacterium erythrogenes* is generally similar to the phenomenon observed with pseudomonads (Barrett *et al.* 1953; Kogut & Podoski, 1953; Clarke & Meadow, 1959), Azotobacter (Karlsson & Barker, 1948; Stone & Wilson, 1952) and *Aerobacter aerogenes* (Davis, 1956). Cell-free preparations from unadapted organisms contain many of the component enzymes of the cycle and since known inhibitors of induced enzyme formation (e.g. chloramphenicol, 2:4-dinitrophenol, azide, increase in temperature; Knox, 1953) all prevented the adaptive process it is reasonable to postulate a permeability barrier in these organisms which may be overcome by inducible transport mechanisms. However, the use of oxidation as an index of permeability, though necessary with metabolizable substrates, is an indirect measurement, and other explanations are not excluded. Some organisms contain distinct though functionally similar enzymes, for example malic dehydrogenase and isocitric dehydrogenase (Cohn, 1956; Markert & Møller, 1959), and it might be possible for exogenous substrates to evoke special mechanisms for their oxidation.

The ability of lysozyme to cause an apparent increase in certain enzymic activities of *Micrococcus lysodeikticus* was noted by Penrose & Quastel (1930). The primary action of lysozyme on *Corynebacterium erythrogenes*, as on other organisms, is probably the hydrolysis of the rigid cell wall (Salton, 1957), which when weakened sufficiently permits the rupture of the cell membrane and free access of a substrate to the cytoplasm. By balancing the internal osmotic pressure with a suitable impermeable substance it has been shown that protoplasts (Weibull, 1953) and spheroplasts retain many of the functions of the whole organism (McQuillen, 1956; Sistrom, 1958). This may explain why adaptation was still shown after lysozyme treatment of *C. erythrogenes* in the presence of sucrose. The result implies that the permeability barrier does not reside in the cell wall or in that component of the wall that is hydrolysed by lysozyme. In *Bacillus megaterium* (Storck & Wachsman, 1957; Weibull, Beckman & Bergström, 1959) and *Azotobacter vinelandii* (Jose & Wilson, 1959) there is a differential distribution of enzymic activities between the pellet and supernatant fluid fractions from lysozyme-treated organisms. While it is not certain that the pellet fraction obtained by centrifugation of organisms treated with lysozyme consisted of cytoplasmic membranes entirely uncontaminated with enzymes from the cytoplasm, nevertheless the absence of malic dehydrogenase and the cytochrome system from the supernatant fluid of lysed *C. erythrogenes* probably means that the permeability barrier and the inducible transport mechanism, at least for malate, is in the cytoplasmic membrane. This accords with the conclusions of Mitchell & Moyle (1956), Davis (1956) and Rickenberg (1957) about the site of the permeability barrier.

The patterns of adaptation to TCA cycle intermediates in the organisms which have been examined in detail vary considerably; Clarke & Meadow (1959) concluded that separate permeability mechanisms may exist for each substrate. The present finding that adaptation of *Corynebacterium erythrogenes* to L-malate makes the organism acquire permeability to succinate faster than does succinate itself, and that previous incubation with D-malate enables rapid oxidation of all three dicarboxylic acids, suggests that these compounds have a common inducible factor involved in their transport. Alternatively, the results could be explained if L- and D-malate were better inducers of specific succinate and fumarate systems than these substrates themselves. The question whether or not each of these compounds has a specific transport process has some bearing on the mechanism of entry into the cells; at present little is known about this. The inhibitory effect of azide on the oxidation of succinate and acetate by *Escherichia coli* grown on these substrates (Goucher, Woodside & Kocholaty, 1954) and of azide and 2:4-dinitrophenol on the oxidation of certain compounds by whole organisms but not by extracts of *C. erythrogenes* probably means that transport is an energy-requiring process, though if this is so it is curious that these two inhibitors had less effect when added after the substrate. This result may indicate that penetration is faster than oxidation, so that there is a temporary intracellular accumulation of substrate. Mitchell (1957, 1959) proposed that succinate, malate, fumarate and other compounds enter bacteria by group transfer reactions, and gave some evidence for the permeation of succinate into *Micrococcus lysodeikticus* as a succinyl compound (Mitchell & Moyle, 1959). The enzymes in extracts of *C. erythrogenes* which appear to be responsible for the formation of hydroxamate compounds with malate and fumarate require further investigation, but their presence in both adapted (induced) and unadapted organisms implies that they do not form the inducible part of the transport mechanism. Their location mainly in the supernatant fluid after centrifugation of extracts does not necessarily mean that they do not function in penetration; vigorous shaking with glass beads is capable of releasing enzymic activity from particulate fractions (Linnane & Still, 1955) and some release possibly occurs during ultrasonic disintegration and after lysis with lysozyme.

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With 1 *plate*
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Differentiation of *Acetomonas* and *Pseudomonas*

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SUMMARY

A comparative study of 50 strains of *Acetomonas* and 165 strains of *Pseudomonas* showed that the two genera could be easily and rapidly differentiated by the use of four criteria; these were: (*a*) production of acid on ethanol + $CaCO_3$ agar plates, (*b*) oxidation of calcium lactate to carbonate, (*c*) production and accumulation of dihydroxyacetone in glycerol media, (*d*) growth at (initial) pH 4·5. *Acetomonas* was positive with (*a*), (*c*) and (*d*), but negative with (*b*); *Pseudomonas* was positive only with (*b*). Liquefaction of gelatin and production of a greenish yellow fluorescent pigment, being variable with *Pseudomonas* and absent from *Acetomonas*, were useful only when positive.

INTRODUCTION

The belief in the universal polar flagellation of all motile acetic acid bacteria seems to have originated from the publication by Zeidler (1898) of a photomicrograph showing a single polar flagellum on several cells of *Termobacterium aceti*. Later, Vaughn (1942, 1943) published two papers with the stated purpose of drawing attention to the insufficiently recognized polar flagellation of all motile acetic acid bacteria, together with a photomicrograph of a cell with a single polar flagellum. These papers became widely known, and probably largely on this account Stanier (1947) was led to say 'In view of the extensive morphological and biochemical parallelism between acetic acid bacteria and organisms of the *P. fluorescens* type, it seems indefensible any longer to maintain a family Acetobacteriaceae; its members should be incorporated in the family Pseudomonadaceae'. At the time this seemed supported by his statement that 'some fluorescent pseudomonads' could apparently produce some acid from ethanol when $CaCO_3$ was present in the medium to neutralize free acid as soon as formed, and to prevent a decrease in pH value, for below pH 5·0 the bacteria rapidly died. It seems probable that the belief in the universal polar flagellation of the acetic acid bacteria became widely accepted for so long that it was thought unnecessary to stain the flagella of motile strains; they seem automatically to have been recorded as possessing 'a single polar flagellum'. Thus in the 6th edition of *Bergey's Manual* (1948) all motile *Acetobacter* strains were so described.

The work of Leifson (1954), confirmed by Shimwell (1958, 1959) and Asai & Shoda (1958), however, has changed the picture. It is now known that motile acetate-oxidizing acetic acid bacteria have peritrichous flagella, and that it is only the motile ones which do not oxidize acetate (the 'suboxydans group' of Frateur, 1950) which have one or more (usually more) polar flagella. Although three years elapsed between Leifson's publication and the 7th edition of *Bergey's Manual* (1957) his findings were apparently not investigated, and the statement regarding the monotrichous polar flagellation of all motile strains of *Acetobacter* was repeated, on the grounds that Leifson's photomicrographs needed confirmation by unequivocal ones, or by electron micrographs, before his proposal to divide the acetic acid bacteria into *Acetobacter* (peritrichously flagellate, fully oxidizing) and *Acetomonas* (polarly flagellate, only partly oxidizing) could be accepted. This has since been done (Shimwell, 1958, 1959; Asai & Shoda, 1958; Shimwell & Carr, 1959), and there is no longer any doubt of the need to divide the heterogeneous acetic acid bacteria into their two component genera. Plate 1, fig. 1, shows the peritrichous flagella of a strain of *Acetobacter rancens*; Pl. 1, fig. 2, the polar ones of *Acetomonas oxydans*, the proposed type-species (Shimwell & Carr, 1959).

Although Stanier (1947) was thus wrong in his insistence that all acetic acid bacteria should be recognized as pseudomonads and transferred from Acetobacteriaceae to Pseudomonadaceae, there still remained, however, the question whether the acetic acid production by some strains of *Pseudomonas fluorescens* constituted sufficient grounds for regarding them as acetic acid bacteria taxonomically indistinguishable from *Acetomonas*; or, conversely, whether *Acetomonas* strains should be included in *Pseudomonas*, there to swell the number of already overcrowded and often ill-defined species. The examination of this point is the main object of this paper.

To those only familiar with either *Acetomonas* or *Pseudomonas* a written list of the properties common to both genera, derived from the literature and specially selected to emphasize resemblances, might suggest that it is justifiable to put the two genera together. To those who have handled and studied members of both genera there seems less possibility of mistaking one for the other. Frateur (1950) enumerated some of the major differences between *Pseudomonas* and his suboxydans group (= *Acetomonas*) such as the inability of the acetic acid bacteria to liquefy gelatin, to show appreciable growth on peptone agar, to produce yellowish green fluorescent water-soluble pigments (admittedly not found in all *Pseudomonas* species), to grow on potato, to metabolize protein and so on. In addition to these negative properties of *Acetomonas*, however, we consider it important to emphasize their outstanding positive properties which are not possessed by *P. fluorescens* and similar strains, and which sharply distinguish these two different types of bacteria.

METHODS

Strains used. These comprised the 50 *Acetomonas* strains previously studied by Shimwell & Carr (1959), and the 165 *Pseudomonas fluorescens* and similar strains studied by Rhodes (1959; excluding *Aeromonas* strains).

Biochemical methods. These were mainly those described by Frateur (1950; some as modified by Shimwell & Carr, 1959), and those of Rhodes (1959). It is convenient to give the methods used to obtain the results in Table 1.

(1) The production of acetic acid from ethanol was detected by the presence on agar plates of 1% (w/v) Difco yeast-extract + 2% (v/v) ethanol + 2% (w/v) $CaCO_3$,

of clear zones of dissolved $CaCO_3$ round the implanted (*c.* 1 cm.^{2}) bacterial masses after incubation for 14 days at 26°, or sooner.

(2) *Growth at* pH 4·5. In the case of *Pseudomonas* strains this was studied in YE liquid medium adjusted to pH 4·5 with HCl (Rhodes, 1959) and in similarly adjusted 1 % (w/v) Difco yeast extract + 2 % (v/v) ethanol, or similar alcoholic media in the case of *Acetomonas* strains, since the latter grow poorly (if at all) on YE liquid medium alone.

(3) *Oxidation of calcium lactate through acetate to carbonate.* This was studied on 1 % (w/v) Difco yeast extract + 1 % (w/v) calcium lactate agar plates, the production of carbonate being shown by the appearance of nodular crystals of $CaCO_3$ under and/or around the implanted bacterial masses (*c.* 1 cm.^{2}) as seen through the bottom of inverted plates at a magnification of × 40.

Production and accumulation of dihydroxyacetone in media containing glycerol. Agar plates containing 1 % (w/v) Difco yeast extract + 2 % (v/v) glycerol were used. After various periods of incubation (1–14 days) at 26°, the plates were flooded, in the cold, with Fehling's solution, a quick (10 min.) production of an aureole of yellow to red copper oxide(s) round the implanted masses indicating the presence of dihydroxyacetone.

RESULTS

The results obtained with the primary differentiating criteria are recorded in Table 1. With the one exception in a total of 215 strains examined any one of the primary differentiating criteria served to eliminate either *Acetomonas* or *Pseudomonas*; any two of these criteria did so without exception (the one apparently anomalous strain will be referred to in the Discussion).

Table 1. *The results of using the four primary differentiating tests with* 50 Acetomonas *and* 165 Pseudomonas *strains*

Test	*Acetomonas* No. strains positive	*Pseudomonas* No. strains positive
(*a*) Production of acetic acid on ethanol + $CaCO_3$ agar	50 (in 2 days)	0 (in 14 days)
(*b*) Oxidation of lactate to carbonate	0	165
(*c*) Dihydroxyacetone from glycerol	*49 (in 18 hr.)	0 (in 14 days)
(*d*) Growth at pH 4·5	50	0

* See Discussion.

There were secondary differentiating criteria which concerned characteristics which were never possessed by the *Acetomonas* strains, but which were usually (but not always) possessed by the *Pseudomonas* strains studied. These were the liquefaction of gelatin and the production of fluorescent yellow-green water-soluble pigment. These secondary differentiating characteristics, when positive, almost certainly rule out *Acetomonas*, but when negative are inconclusive. Other similar secondary criteria (e.g. peptonization of milk, etc.) are excluded as superfluous.

DISCUSSION

It is submitted that *Acetomonas* can readily be distinguished from *Pseudomonas*; indeed, it is virtually impossible to confuse them. We submit also that the differences shown in Table 1 are adequate and suitable for decisive, easy and rapid laboratory differentiation. Moreover, when these primary characters are considered in conjunction with the secondary characteristics, together with those listed by Frateur (1950) and by Rhodes (1959), it seems that there are relatively few resemblances between the genera *Acetomonas* and *Pseudomonas* apart from polar flagellation and an oxidative type of metabolism.

With regard to the one strain of *Acetomonas* which did not give dihydroxyacetone from glycerol, it was reported (Shimwell & Carr, 1959) that this strain (*A. suboxydans*, NCIB 3734) also differed from the remaining 49 strains in not producing brown water-soluble pigment from any sugar used. As it was also non-motile, its differentiation from non-motile *Acetobacter* strains had to be based solely on its inability to oxidize acetic acid, lactate or acetate. It was suggested, however, (Shimwell, 1957) that in view of the facile mutability in *Acetobacter*, it was conceivable that even the ability to oxidize acetate might be capable of loss through mutation; and that if this were so, such a mutated *Acetobacter* strain might if non-motile be difficult to distinguish from a non-motile *Acetomonas* strain. We think that the anomalous strain in question may be an example of this possible situation. However, there is no question of the organism NCIB 3734 being a *Pseudomonas* strain, for it is a strong producer of acetic acid from ethanol, grows readily at pH 4·5, and does not oxidize acetic acid, acetate or lactate.

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EXPLANATION OF PLATE

Fig. 1. *Acetobacter rancens* strain. Electron micrograph showing peritrichous flagella. × 15,000.

Fig. 2. *Acetomonas oxydans*, cider strain. Electron micrograph showing multitrichous polar flagella. × 15,000.

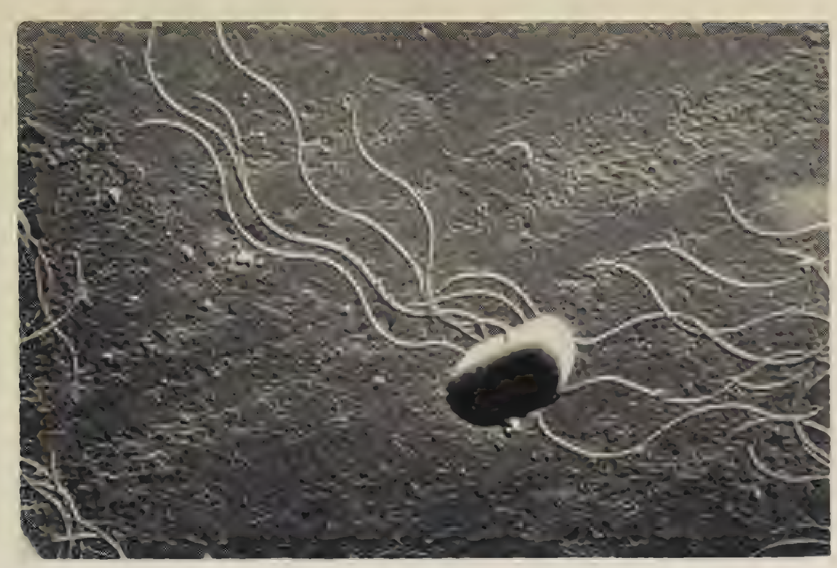

Fig. 1

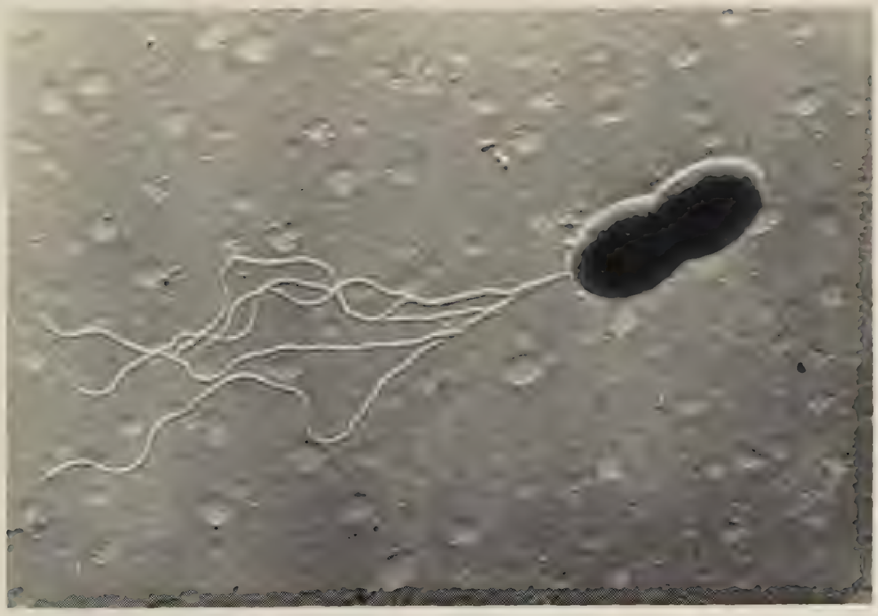

Fig. 2

J. L. SHIMWELL, J. G. CARR AND M. E. RHODES (*Facing p. 286*)

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The Dissociation of Morphogenesis from Cell Division in the Cellular Slime Mould, *Dictyostelium discoideum*

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SUMMARY

Dictyostelium discoideum, strain Fty-1, myxamoebae were harvested from the stationary phase and dispensed in small population samples on washed agar. Total cell counts were made at intervals between deposition and fruit construction. Within an uncertainty level of 2·5 %, the data indicate that the populations remained constant and the terminal fruits contained the same number of cells as had formed the aggregates. Subsidiary evidence based on viability determinations and the known morphology of moribund cells suggested that new cells did not arise nor old cells die.

When myxamoebae were harvested from the log phase while still feeding on a high density of bacteria, a substantial degree of cell division did occur during morphogenesis. But the increase in cell number of myxamoebae which had not aggregated corresponded approximately, with that of myxamoebae which had aggregated and fruited. Consequently such increases appear to be coincidental with and not causally related to the morphogenetic sequence.

INTRODUCTION

Growth and morphogenesis can occur separately in the development of the slime moulds if by growth is meant an increase in protoplasmic mass. This is supported by the following observations. Washed myxamoebae on washed agar gel display the normal morphogenetic pattern (Sussman & Nöel, 1952). Since effectively no exogenous nutrients are present, a net increase in protoplasmic mass is impossible. Myxamoebae, harvested at any stage of the log phase and washed free of bacterial associates, can aggregate and construct normal fruits without unusual delay (Sussman, 1956). Yet, when left untouched on the growth plate, these myxamoebae would not have begun morphogenesis until the bacterial food supply had been exhausted and the stationary phase attained. The addition of fresh bacteria to myxamoebae which have completed an early stage of the morphogenetic sequence brings about its cessation and their return to the vegetative stage (Raper, 1941).

It had long been assumed that morphogenesis is uncoupled not only from protoplasmic increase but also from cell division (Raper, 1941). Subsequently, however, Bonner & Frascella (1952) reported mitotic divisions in *Dictyostelium discoideum* pseudoplasmodia, beginning at the anterior end and proceeding posteriorly in a wave. The peak incidence of metaphase figures was *c.* 2 % which, because cell division is accomplished rapidly, reflected an appreciable increase in cell number.

These results suggested the possibility that cell division might play a role more than coincidental in the morphogenetic sequence. Wilson (1952, 1953), Wilson & Ross (1957) and Ross (1959) also reported division figures during fruit construction by *Dictyostelium discoideum* and concluded that both meiosis and mitosis occurred and further, that syngamy by cell engulfment occurred immediately before aggregation. The fact, then, that one can actually observe cell division during fruiting has made it important to determine whether there is an obligatory relation between the two. An examination of this question has been carried out with a 'Fruity' mutant of *D. discoideum*, strain Fty-1 (Sussman, 1955). This mutant can form tiny aggregates, composed of very few cells; this made it possible to obtain accurate cell counts at all morphogenetic stages. The results indicate that normal construction can go on without cell division. One can observe considerable increases in cell numbers during aggregation and fruiting by harvesting cells while they still contain a large endogenous supply of bacteria. However, such cells continue to divide independently of whether or not they happen to be engaged in constructing a fruit.

METHODS

Organism. Dictyostelium discoideum strain Fty-1 was grown on SM agar in association with *Aerobacter aerogenes* (Sussman, 1951). This strain is a mutant of *D. discoideum* NC-4, obtained by ultraviolet irradiation. Chromosome counts of preparations fixed by osmium tetroxide, and stained with aceto orcein, showed it to be a stable diploid stock (Ross, 1959). In contrast, the wild type from which it was derived has been found to be haploid. After incubation for 40–44 hr. at 22°, the myxamoebae had attained the stationary phase and were harvested, washed by differential centrifugation and dispensed in drops from a micropipette on to washed water agar gel.

Counting procedures. Two counts were made of each population sample during the pre-aggregative period; precision analysis is given in the next section. After fruiting, the fruits were examined *in situ* under oil at × 950 magnification to count the spores, stalk- and basal-disk cells (Sussman, 1955). Aggregates had first to be dispersed before cell counts could be made; this was accomplished by trypsinization.

Aggregate dispersal. The trypsinization procedure used was that of Trinkaus & Groves (1955). A drop of buffered trypsin solution was placed on the aggregated cells. After a few minutes, by which time the excess fluid had been absorbed by the agar, the aggregated myxamoebae were completely dispersed and could be counted precisely.

RESULTS

The precision of the counting procedure

Tables 1 and 2 are a summary of the data concerned with the precision of the counting; counts were made immediately after deposition of the cells on washed water agar gel and again after incubation for 3 hr. The data from experiment 4-4 are given in Table 1 and summarized with those of three other experiments in Table 2. In experiment 4-4, the *mean percentage difference* between the successive counts was 0·3 cells/drop or 1·6 %. The highest in any of the experiments was 2·3 %.

Cell turnover

Direct counts can detect only net changes in cell number. Thus, a system in which new cells appeared at an appreciable rate, but old cells died and lysed immediately at the same rate would reflect no change in number. That no such dynamic equilibrium played a part in the experiments to be described is indicated by the following observations. Moribund myxamoebae on washed agar did not lyse and disappear immediately. Instead, they swelled to gigantic size, became perfectly smooth in outline and devoid of internal structure. After an hour or so they lysed but ghosts remained in the form of the collapsed cell membranes and granular debris. Thus an appreciable number of cells could not have died, nor new cells have appeared, without making this fact apparent. Since the cells did not lyse immediately, an appreciable death rate, had it existed, should have been detectable by a difference between direct counts and viable counts. To test this, several microdrops were dispensed

Table 1. *Precision of direct counts*

Myxamoebae were dispensed in microdrops on washed water agar gel. Cell counts were made at 0 hr. (A) and again after 3 hr. incubation (B).

Experiment 4–4

Number of cells			
A 0 hr.	B 3 hr.	B–A	Difference %
18	17	−1	2·9
18	16	−2	5·9
23	23	0	0
24	24	0	0
25	23	−2	4·2
30	28	−2	3·5
37	39	2	2·6
39	40	1	1·3
48	48	0	0
51	51	0	0
51	50	−1	1·0
55	55	0	0
59	56	−3	2·6
60	60	0	0
61	64	3	2·4
63	63	0	0
68	68	0	0

Mean % difference = 1·6.

Table 2. *Determinations of the precision of the cell-count procedure*

Data from four separate experiments are summarized, all performed as described in Table 1.

Experiment	No. drops	Mean no. cells	Smallest and largest samples	Mean % difference
3-5	18	46	30–70	2·1
3-12	22	41	30–66	0·8
3-20	14	42	16–70	2·3
4-4	17	43	18–68	1·6

singly, on plates of washed water agar gel and direct counts were made immediately and after incubation for 18 hr. Then a few drops of a broth culture of *Aerobacter aerogenes* were added and the myxamoebae and bacteria distributed over the agar surface with a flamed glass spreader. After 2–3 days, plaques of myxamoebae appeared within the film of bacteria (Sussman, 1951). Table 3 summarizes the data and shows that direct and viable counts were in good agreement. Further, past studies of *Dictyostelium discoideum* wild type and 'aggregateless' mutants have indicated that myxamoebae can remain 100 % viable and constant in number on washed water agar gel for periods up to 96 hr. (Sussman, 1952).

Table 3. *Viability determinations*

Cell counts were made within microdrops on washed agar. Then bacteria were added and plaques were allowed to develop and were counted as described in the text.

Cell count	Plaque count	% viability
5	5	100
6	6	100
6	7	114
11	11	100
12	11	91
13	13	100
14	14	100
18	17	94
21	19	90
27	26	96
36	34	94
54	50	93
223	213	96

Direct counts after aggregation and fruit construction

Microdrops were dispensed on to washed water agar gel. Counts were made immediately and after incubation for 3 hr. At 18–24 hr. counts were made of drops which had not aggregated. Simultaneously some aggregated populations were dispersed by trypsinization and counted. Others were allowed to complete construction of the fruiting body and these were counted under oil as previously described. Table 4 gives a summary of the data. As seen, the net changes in cell number were negligible yielding a mean difference of about 2·5 %, a value indistinguishable from the fluctuations due to the counting error shown in Table 1. Thus, under these conditions and within the limits of the counting procedure, normal aggregation and fruit construction were completely dissociated from changes in cell number.

The experiment summarized in Table 5 shows that, when the myxamoebae possessed considerable endogenous stores of undigested bacteria, fruit construction was accompanied by considerable cell division. Myxamoebae were harvested from growth plates before all the bacteria had disappeared. The myxamoebae were washed by centrifugation to free them of external bacteria (Sussman, 1952) but microscopic examination revealed the presence of large numbers of undigested intracellular bacteria. Microdrops were dispensed on washed water agar gel. Cell counts were made immediately and after incubation for 18 hr. in drops containing no aggregates, and after 30 hr. in drops containing aggregates, that is, after terminal

Table 4. *Changes in cell number during aggregation and fruiting*

I			
A	B		
0 hr.	18 hr.	B − A	% difference
12	13	1	4·0
17	18	1	2·9
17	17	0	0
19	17	−2	5·6
20	19	−1	2·6
21	20	−1	2·4
22	25	3	6·4
23	23	0	0
25	26	1	1·9
25	24	−1	2·1
26	28	2	3·7
27	27	0	0
32	32	0	0
35	36	1	1·4
40	40	0	0
48	48	0	0
50	46	−4	4·2
53	57	4	3·6
Net change/drop			0·2
Mean % deviation			2·3

II					
A	B				
0 hr.	Out	In	Total	B − A	% difference
14	1	13	14	0	0
21	11	10	21	0	0
22	9	11	20	−2	4·8
25	7	20	27	2	3·9
25	13	12	25	0	0
25	0	27	27	2	3·8
29	9	24	33	4	6·5
30	22	14	36	6	9·1
31	15	16	31	0	0
32	3	31	34	2	3·0
32	8	22	30	−2	3·2
34	11	22	33	−1	1·5
35	4	25	29	−6	9·4
35	6	30	36	1	1·4
36	29	10	39	3	2·7
38	5	33	38	0	0
38	3	38	41	3	3·8
39	10	30	40	1	1·7
39	29	10	39	0	0
44	14	28	42	−2	2·3
48	15	34	49	1	1·0
50	30	22	52	2	2·0
54	27	24	51	−3	2·9
57	24	32	56	−1	0·9
72	28	44	72	0	0
72	13	60	73	1	0·7
75	26	49	75	0	0
Net change/drop					0·4
Mean % deviation					2·4

III						
A	B					
0 hr.	Out	Stem	Spore	Total	B − A	% difference
26	11	7	9	27	1	1·9
47	14	6	21	41	−6	6·8
50	10	7	28	45	−5	5·3
50	10	6	34	50	0	0
51	17	6	31	54	3	2·8
52	15	7	33	55	3	2·9
53	26	9	19	54	1	0·9
62	34	6	20	60	−2	1·6
63	19	7	33	59	−4	3·3
66	13	7	46	66	0	0
68	15	9	48	72	4	2·9
71	26	9	37	72	1	0·7
87	13	7	65	85	−2	1·2
30	6	4	20	30	0	0
38	17	3	22	42	4	5·0
35	4	5	30	39	4	5·4
Net change/drop						0·1
Mean % deviation						2·6

I. Drops which did not aggregate; counted at 0 hr. and 18 hr.
II. Drops which aggregated and were dispersed by trypsin treatment at 18 hr. ‘Out’ and ‘in’ refer to the cells which were outside and inside the aggregates.
III. Drops which aggregated and fruited. Counts were made at 28 hr. ‘Out’ refers to cells outside the fruit. ‘Stem’ refers to cells in both the stem and basal disk.

fruits had appeared. In the latter class, an increase of 29 cells/drop was observed for an average of almost 33 %. The increase was not as great in drops without aggregates but probably equivalent when it is considered that these counts were made 12 hr. earlier. Thus it appears that the cell division here encountered bore no causal relation to the fruiting process but merely reflected the extraordinary endogenous food reserves possessed by the myxamoebae when these are actively feeding upon a dense bacterial population. Had the myxamoebae been allowed to grow upon a less dense bacterial population (Sussman, 1956) or to attain the stationary phase before harvesting so as to deplete the quantity of intracellular undigested bacteria, the extraneous cell division would have been eliminated as it was in the experiments shown in Table 4.

Table 5. *Changes in cell number during fruiting*

I				II						
A	B		%	A	B					%
0 hr.	18 hr.	B–A	difference	0 hr.	Out	Stem	Spores	Total	B–A	difference
16	25	9	22	46	14	10	35	59	13	24·8
21	27	6	12·5	52	31	8	30	69	17	28
30	54	24	31	69	17	5	72	94	25	30·6
33	45	12	15·4	70	22	5	74	101	31	36·3
37	43	6	7·5	74	39	7	93	139	65	61
38	54	16	17·4	74	20	15	54	89	14	17·1
45	70	25	21·8	82	38	7	77	122	40	39·1
46	63	17	15·6							
47	59	12	11·3							
46	64	18	16·3							
Net change/drop			14·5					29·3		
Mean % difference			17·1					32·8		

I. Drops which did not aggregate; counts at 0 and 18 hr.

II. Drops which fruited. Counts made at 0 and 30 hr. 'Out' refers to cells outside the fruit. Stem' refers to both stem and basal disk cells.

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Yeasts from Antarctica

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SUMMARY

The yeast flora of moss from Granite Harbour and of soils from Wright Dry Valley in the McMurdo Sound region of Antarctica was examined. There were 75,000 yeasts/g. on the moss; species recovered were *Cryptococcus laurentii*, *C. albidus* and *Rhodotorula minuta*. Yeasts were isolated from 8 of the 12 soil samples in numbers up to 5000/g. Strains of *Candida scottii*, whose maximum growth temperature was *c.* 15°, were the predominant soil yeasts.

INTRODUCTION

The soils of Antarctica are characteristically very cold and dry. Although some have recognizable pedological features (personal communication from J. D. McCraw), all have a primitive organic cycle, for the terrestrial photosynthesizing vegetation of the continent is virtually limited to algae, mosses and lichens. Because of the climatically extreme conditions and the sparse macro-flora and -fauna, the microbiology of Antarctic soils is peculiarly interesting. The following is an account of yeasts recovered from soils and moss collected in the region of McMurdo Sound.

MATERIALS

In February 1957 some moss (*Bryum antarcticum*) with sand adhering to it was collected from a rock crevice 20 ft. above tide level at Granite Harbour, McMurdo Sound. It was sent to New Zealand by air and reached the laboratory 7 days after collection. The sample weighed *c.* 500 g. and its pH value was 6·5.

Seven samples of soil containing algae (No. 474, 476, 478, 479, 482, 483, 487) were collected from Wright Dry Valley, McMurdo Sound, in January 1958. They were taken with sterile precautions and kept in deep freeze until they reached the laboratory.

Five other soil samples were collected in January 1959, also in Wright Dry Valley. Their descriptions are as follows: (1) Wet granite sand at edges of pools east of Lake Vanda; pH 8·0. (2) Encrustation from depression on floor of North Arm, Wright Dry Valley; pH 8·5. (3) 'Loess'-like sand from granite basement between dolerite dyke swarms; pH 8·2. (4) Sand from centre of a damp depression, the upper layer appearing to have an organic component; pH 8·0. (5) Sand from a damp depression, basement granite with dolerite dykes, organic debris on surface; pH 8·1. These samples were also taken with sterile precautions and kept refrigerated.

METHODS

The moss sample was cultured on acidified glucose peptone agar plates (glucose, 4 %, w/v; peptone, 1 %, w/v; agar, 2 %, w/v; brought to pH 4–4·5 by the addition of 10 % (v/v) HCl solution to the molten medium immediately before the plates were poured; the solidified plates were dried in an oven at *c.* 70° before inoculation so that the water in the inoculum would be absorbed). Ten g. moss were diluted serially with sterile tapwater to 1/25, 1/250, 1/1000 and 1/10,000. Inocula of 0·5 ml. were spread on the surfaces of the plates and 6 plates inoculated from each dilution. Four plates/dilution were incubated at room temperature and two at 4°.

Three of the seven 1958 soil samples (No. 474, 482, 487) were cultured on acidified glucose peptone agar. As the samples were small, dilutions were made from amounts varying between 1·8 and 3·5 g. to 1/100, 1/1000 and 1/10,000. Five plates were inoculated from each dilution; three plates/dilution were incubated at room temperature and two at 4°.

The five 1959 soil samples were cultured on acidified glucose peptone agar and on aureomycin + glucose peptone agar (100 mg. aureomycin/l. glucose peptone agar. Dilutions were made from 10 g. soil to 1/10, 1/100 and 1/1000. Eight plates of acidified glucose peptone agar and four plates of aureomycin + glucose peptone agar were inoculated from each dilution of each sample. In addition, 1 ml. of each dilution of the five samples was inoculated into two flasks each containing 100 ml. of nitrogen-depleted broth (glucose, 5 g.; KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; Difco Bacto yeast extract, 0·1 g.; double glass-distilled water, 1 l.). Half of these cultures (four acidified glucose peptone agar plates, two aureomycin + glucose peptone agar plates, one flask nitrogen-depleted broth for each dilution of each sample) were incubated at room temperature and half at 4°.

Cultures held at room temperature were incubated for 3–7 days before counts were made and subcultures taken. The moss cultures held at 4° were incubated for 11 days, the 1958 soil cultures for 4 weeks and the 1959 soil cultures for 6 weeks. Both sets of cultures in nitrogen-depleted broth (those at room temperature and those at 4°) were subcultured after 6 weeks incubation. Two pour plates were made from each flask (1 ml. inoculum/plate), one with the nitrogen auxanographic solid medium of Lodder & Kreger-van Rij (1952, p. 26), one with the same medium + one drop 0·1 % (w/v) yeast extract/plate. Plates inoculated from flasks incubated at room temperature were incubated at room temperature; those from flasks incubated at 4° were incubated at 4°. Colonies were picked from the room temperature plates after incubation for 3 weeks, and from the 4° plates after 7 weeks.

RESULTS

The temperature of incubation did not affect the numbers and species patterns of yeasts isolated from the moss sample. The room temperature cultures indicated that there was a population of 75,000 yeasts/g. wet weight, the 4° cultures one of 70,000. Fifty isolates were subcultured from the room temperature cultures; 72 % were *Cryptococcus albidus* (Saito) Skinner, 20 % *C. laurentii* (Kufferath) Skinner, and 8 % *Rhodotorula minuta* (Saito) Harrison. Forty-five isolates were subcultured from the 4° cultures; all of these grew at least as readily at room temperature as at 4°; 75 % were *C. albidus*, 18 % *C. laurentii* and 7 % *R. minuta.*

There was no yeast growth at room temperature on the glucose peptone agar cultures of the 1958 soil samples 474, 482 and 487; at 4° there was growth of yeasts on the cultures of soil 487 at the rate of 5000 yeasts/g. wet weight, but no growth on cultures of 474 and 482.

All seven of the 1958 soils had been cultured for streptomyces in a non-quantitative manner on casein agar (Vernon, 1958) and incubated at 4°. No streptomyces appeared but there was yeast growth on plates inoculated with soils 474, 476, 478 and 487. Presumably yeasts occurred in soil 474 at a rate of less than 100/g., the lowest number that the quantitative glucose peptone agar cultures would detect. Fifty yeasts were subcultured from the glucose peptone agar cultures of soil 487 and eleven from the casein agar. Eleven yeasts were recovered from the casein agar cultures of soil 474, two from 476 and ten from 478. All but three of these isolates were obligatory psychrophilic strains of *Candida scottii* Diddens & Lodder. One of the two isolates from soil 476 was a strain of *Rhodotorula mucilaginosa* (Jorg.) Harrison which grew well at room temperature. Two of the isolates from soil 487, one recovered from glucose peptone agar and one from casein agar, resembled *C. scottii* except that they could not use potassium nitrate as a nitrogen source.

The yeasts isolated from the five 1959 soil samples cultured on solid media are shown in Table 1. No yeasts were isolated from soil 2 and numbers in the other soils were low. Using the counts from the acidified glucose peptone agar plates incubated at 4° as a basis for calculation, there were 20 yeasts/g. in soil 1, 40 in soil 4 and 30 in soil 5. Yeasts were isolated from soil 3, but the only colony found on the acidified glucose peptone agar plates was on a plate inoculated from the 1/1000 dilution. Psychrophilic *Candida scottii* was present in soils 1, 4 and 5 together with a number of *Cryptococcus* and *Rhodotorula* spp. *Sporobolomyces odorus* Derx and *Cryptococcus* spp. were isolated from soil 3.

Moulds were present in some of the samples examined; they grew more vigorously on plates incubated at room temperature than at 4°. There were 450,000 mould colonies/g. moss on plates held at room temperature; yeasts on these plates were counted and subcultured earlier than was desirable because of the spreading habit of the moulds. On moss plates at 4° the relative growth rate of the yeasts was greater than that of moulds, which at this temperature had restricted, submerged colonies. There were no moulds in the 1958 soils 474 and 487, but 1600/g. in soil 482. In the 1959 soils, using the counts on aureomycin + glucose peptone agar at room temperature, there were 15 moulds/g. in soil 1, none in soil 2, 20 in soil 3, 10 in soil 4 and 20 in soil 5.

Growth in nitrogen-depleted broth subcultured into auxanographic agar was largely of yeasts and moulds; bacterial colonies were only occasional. There were many small yeast and mould colonies in plates from nitrogen-depleted broth flasks inoculated with 1/10 dilutions of soils 1, 4 and 5, and with 1/100 dilutions of soils 4 and 5, but only a few colonies on other plates. Addition of yeast extract to the auxanographic agar made no apparent difference in amount and nature of growth. The following yeasts were recovered from nitrogen-depleted broth at room temperature: *Cryptococcus albidus* from soils 1, 3, 4 and 5; *C. diffluens* (Zach) Lodder & Kreger-van Rij from soil 1; *Rhodotorula glutinis* (Fres.) Harrison from soils 1 and 2; *R. mucilaginosa* from soil 2; a non-fermenting *Torulopsis* sp. from soil 2. The yeasts recovered from nitrogen-depleted broth at 4° were: *C. luteolus* (Saito) Skinner from soil 5; *Candida scottii* from soils 4 and 5; *R. glutinis* from soils 3 and 4; *R. texensis* Mrak, Phaff & Williams from soils 1, 2 and 5; a cream, fluid, psychrophilic yeast, which resembled *C. scottii* but which could not be freed from a contaminant mould for identification, from soil 1.

Table 1. *Yeasts isolated on acidified glucose peptone agar* (pH 4 *GPA*) *and on aureomycin + glucose peptone agar* (*aureomycin GPA*) *from five soils from Wright Dry Valley, McMurdo Sound. No yeasts were recovered from soil* 2

	Soil 1		Soil 3		Soil 4		Soil 5	
	pH 4 GPA	Aureomycin GPA	pH 4 GPA	Aureomycin GPA	pH 4 GPA	Aureomycin GPA	pH 4 GPA	Aureomycin GPA
Numbers of isolates at room temperature								
Sporobolomyces odorus	.	.	1	1	.	1	.	1
Cryptococcus laurentii	.	.	1	.	.	.	1	.
C. albidus	1	.	1	.	.	.	.	.
C. luteolus	.	1	.	.	.	.	.	.
C. diffluens	.	.	.	.	.	1	.	.
Candida scottii	.	.	.	.	.	.	.	2
Rhodotorula minuta	.	.	.	.	.	.	1	.
R. graminis di Menna	1	.	.	.	.	.	.	.
Total number of isolates	2	1	3	1	0	2	2	3
Numbers of isolates at 4°								
Sporobolomyces odorus	.	.	1	.	.	.	.	.
Cryptococcus laurentii	2	1	.	.	.	.	.	.
C. diffluens	.	.	.	1	.	.	.	.
Candida scottii	1	.	.	.	6	1	6	4
Rhodotorula minuta	1	.	.	.	1	1	.	.
Total number of isolates	4	1	1	1	7	2	6	4

Characteristics of Candida scottii *isolates*

As all strains of *Candida scottii* isolated were obligate psychrophils, their identification presented some difficulties. Representative cultures on glucose peptone agar slopes were incubated at various temperatures. Growth was apparent in 4 days at 0° and appeared to be as rapid at 4° as at 10°. At 15–16° half of the 18 isolates used showed no growth after 10 days, and in the other half growth was scant, dull and wrinkled, not copious and mucoid to fluid as at lower temperatures. Cultures held at 20° for 10 days showed no growth but remained viable. Cultures held at room temperature for 20 days in winter showed poor growth of the same type seen in cultures at 15°. Ability to produce limited growth at room temperature in winter explains the presence of two isolates of *C. scottii* in room temperature cultures of soil 5 (Table 1). Although the 1959 soils were collected in January they did not reach the laboratory until May. Both the above isolates grew well at 4°. All subcultures used in identification were incubated at 4° and held for at least 4 times the period used at room temperature; i.e. liquid auxanograms (solid auxanograms were usually unsatisfactory) were kept for 3 months. Apart from the lower maximum temperature for growth, the Antarctic isolates of *C. scottii* resembled those described by Lodder & Kreger-van Rij (1952, p. 573). Pseudomycelium was well developed; there was no fermentation; glucose, sucrose, maltose, and galactose were used, but not lactose; potassium nitrate was used as a nitrogen source. In addition to these diagnostic characters, these isolates of *C. scottii* liquefied gelatin within 10 days and were able to grow in a vitamin-free medium.

DISCUSSION

If the Antarctic moss with sand adhering to it be regarded as a combination of non-woody plant and soil, the yeasts recovered from it are similar to those found in comparable material in New Zealand. Yeasts from a turf of New Zealand pasture are usually, in the soil, a nitrate-using *Cryptococcus* sp. such as *C. albidus* and, on the leaves of the pasture plants, *C. laurentii* and *Rhodotorula* spp. (di Menna, 1958, 1959*a*).

Candida scottii, which appears to be the predominant yeast in those soils of the Wright Dry Valley which contain organic matter, shows adaptation to habitat. Although the strains studied by Lodder & Kreger-van Rij (1952) would grow at 22°, some of them grew only poorly, so it seems that Antarctic conditions have selected the more psychrophilic strains of a thermophobe species. *C. scottii* is the predictable yeast species to be found in this ecological niche, for it was first isolated by Scott (1936) from chilled beef, and it has the characteristics of a typical soil yeast, being colourless, mucoid, non-fermenting, nitrate-using, and independent of external sources of growth factors (di Menna, 1958).

Although some of the isolates of *Cryptococcus* and *Rhodotorula* spp. recovered from the soil samples, particularly those which occurred on plates inoculated from the higher dilutions, may have been laboratory contaminants, the presence of members of these genera in the moss sample shows that they can indeed be Antarctic inhabitants.

It was not thought that any of the yeasts recovered from nitrogen-depleted broth cultures were capable of fixing atmospheric nitrogen, for yeasts can grow in media with high C:N ratios (di Menna, 1959*b*). Some isolates of *Candida scottii*, inoculated in nitrogen auxanographic liquid media, were left in a refrigerator containing stock cultures on various media. After 5 months, growth in the negative control tubes (basal medium containing glucose, KH_2PO_4, $MgSO_4$, $CaCl_2$, NaCl, growth factors) was as heavy as in the positive controls (same basal medium $+ (NH_4)_2SO_4$) or in the tubes of basal medium $+ KNO_3$. The same isolates were again cultured in nitrogen auxanographic liquid medium and incubated for 5 months, but in a refrigerator which contained no other cultures. There was then no growth in the negative controls. Presumably, during the long incubation period, traces of gaseous nitrogenous compounds produced by the stock cultures in the first refrigerator had contaminated the negative control tubes. The amount of growth in plates from nitrogen-depleted broth cultures probably indicates the relative amounts of nitrogenous material in the five 1959 soils, and suggests that there was more nitrogen in soils 1, 4 and 5 than in soils 2 and 3, and ten times more nitrogen in soils 4 and 5 than in soil 1.

Cultures of *Candida scottii*, AL25, isolated from soil 487, have been deposited at the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, Netherlands. and at the Brewing Industry Research Foundation, Nutfield, Surrey, England.

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The Keto Acid Metabolism of *Corynebacterium diphtheriae* growing in Submerged Culture

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SUMMARY

The keto acid production of *Corynebacterium diphtheriae* (strain CN2000) growing in submerged culture in complex medium was investigated. Pyruvic acid production followed a pattern in which there was a very rapid production of acid which reached a peak about 12 hr. after inoculation. The acid concentration decreased rapidly after 12 hr. and the final pyruvic acid concentration appeared to be very variable. The source of the pyruvic acid is believed to be lactic acid, the concentration of which decreased rapidly as pyruvic acid increased. The presence in the medium of sufficient iron to abolish toxin production completely did not affect the pattern of pyruvic acid production. α-Ketoglutaric acid production in the cultures appeared to vary in a random manner. *C. diphtheriae* strain G12/6 produced a maximum yield of pyruvic acid after only 6 hr. growth but was otherwise similar to strain CN2000. *C. diphtheriae* strain SM1 gave a maximum production of pyruvic acid after 24 hr. but in the presence of 3 μg. Fe/ml. production was more rapid and was like that found for strain CN2000. No correlation was found between toxin production and the production of pyruvic acid or α-keto glutaric acid.

INTRODUCTION

In 1958 Yoneda & Ishihara showed that when they grew the SM1 strain of *Corynebacterium diphtheriae* in static culture for 4 days at 32–33° the addition of increasing amounts of iron to the culture inhibited the formation of toxin and of pyruvic acid. This seemed to be one of the few if not the only instance of a direct relationship between toxin production and a metabolite which had been reported for this organism, and it was decided to investigate the keto acid metabolism of various strains of *C. diphtheriae* growing in a stirred and aerated culture of the type described by Linggood, Matthews, Pinfield, Pope & Sharland (1955).

METHODS

Organisms. Three strains of *Corynebacterium diphtheriae* were used: CN2000 (the Wellcome Research Laboratories Culture Collection); G12/6 supplied by Dr G. D. Clarke; SM1 supplied by Dr M. Yoneda. In each case a 24 hr. growth on a Loeffler slope was used to inoculate 200 ml. medium in a 1000 ml. Erlenmeyer flask.

The flask was placed on a rotary shaker in an incubator at 35° and shaken for 24 hr. after which time the culture was used to inoculate the tank.

Culture vessels and medium. Tanks (20 l.) were used as described by Edwards & Seamer (1960) and contained papain digest of beef (11 l.; Ramon, Pochon & Amoureux, 1941) which had been treated with yeast (Pope & Smith, 1932) and to which 0·5 % (v/v) glacial acetic acid and 2·4 % (w/v) maltose were added. Antifoam A (Midlands Silicone Ltd., Upper Brook Street, London) dispersed in butyl acetate was added (15 ml. 25 %, w/v); air was passed through the tanks (at 500 ml./min.); the temperature was maintained at 35° and the cultures incubated for 48 hr.

Keto acid estimations. Estimations of pyruvic acid and of α-ketoglutaric acid were carried out by a chromatographic method (Cavallini & Frontali, 1954; Cavallini & Mandovi, 1957). The solvent used was a mixture of *n*-butanol, ethanol and 0·5 N-ammonia (70 + 10 + 20, El Hawary & Thomson, 1953). Besides pyruvic acid and α-ketoglutaric acid two other keto acid hydrazones were normally found on the chromatograms (see Jännes, 1954). Since these were unidentified and the pattern of their production was in no way noteworthy they are not further discussed in this paper. On a few occasions a spot corresponding to oxaloacetic acid was also found.

Lactic acid estimations. These were carried out by the method of Barker & Summerson (1941). Since the samples were known to contain large amounts of keto acids, the deproteinized material was treated with 2:4-dinitrophenyl hydrazine and then extracted with benzene before the usual copper sulphate and calcium hydroxide treatment.

Toxin. This was estimated by the flocculation method (Ramon, 1922; Glenny & Okell, 1924). Results are expressed as Lf units/ml. (Lf/ml.).

RESULTS

Experiments with strain CN 2000. Since strain CN 2000 of *Corynebacterium diphtheriae* is the one in use for the routine production of high titre diphtheria toxin at these laboratories it was the one most studied. In all, nine complete analyses were performed in which samples were analysed every 6 hr. during the whole 48 hr. of the incubation period; three of the cultures contained sufficient iron to give complete inhibition of toxin production. The results were subject to variation but in most cases curves of the type shown in Fig. 1 were obtained. Pyruvic acid was produced with great rapidity and by the 12th hr. concentrations higher than 600 μg./ml. were common. This period of production was usually followed by a period of rapid disappearance of the acid. The variations in production were of two types. In the first the height of the peak was much lower than usual (*c.* 300 μg./ml.) and in the second the disappearance of pyruvic acid was relatively slow. Figure 1 also shows the production of α-ketoglutaric acid. It was very variable in the cultures studied and in the examples in Fig. 1 the final α-keto glutaric acid concentrations after 48 hr. growth were 30 and 304 μg./ml. and the corresponding toxin titres were 345 and 305 Lf/ml.

It seemed possible that the source of the pyruvic acid in the cultures might be lactic acid. Tank contents were analysed and the results obtained during the first 24 hr. are shown in Fig. 2. The interpretation of this seems clear. Lactic acid, present in the medium and presumably derived from the beef used in the preparation of the medium, was rapidly converted to pyruvic acid which was then further metabolized and perhaps partially converted back to lactic acid. The disappearance of the pyruvic acid is complicated by the fact that metabolism, presumably via acetate and the tricarboxylic acid cycle, will also be occurring and thus the sum of lactic and pyruvic acids, which in the very early stages of growth is sensibly constant, showed a marked decline.

Since Yoneda & Ishihara (1958) had shown an effect of iron on pyruvate formation it might have been anticipated that the presence of enough iron to inhibit toxin production completely would also exert a marked effect on pyruvic acid metabolism. The results shown in Fig. 3 show that this was not the case. The two curves differ only in the amount of pyruvic acid found after 12 hr., the pattern of metabolism remaining unchanged. Since the toxin yields produced by the two cultures were 340 and 0 Lf/ml., it is clear that pyruvic acid metabolism and toxin production were not closely linked, at least with the CN 2000 strain.

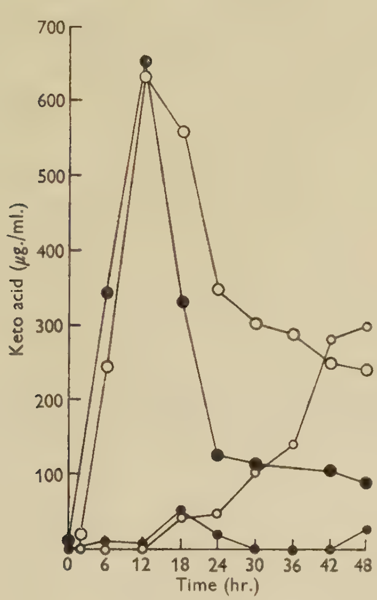

Fig. 1

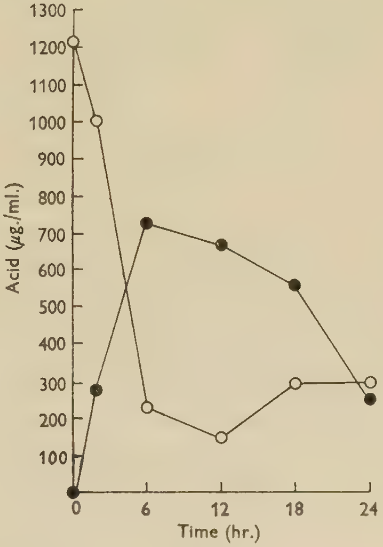

Fig. 2

Fig. 1. Keto acid production in two cultures of the CN 2000 strain of *Corynebacterium diphtheriae.* The 0 hr. samples were uninoculated medium. SC 1272 ○—○, pyruvic acid; ∘—∘, α-ketoglutaric acid. SC 1257 ●—●, pyruvic acid; •—•, α-ketoglutaric acid.

Fig. 2. Lactic acid and pyruvic acid in a culture (SC 1597) of the CN 2000 strain of *Corynebacterium diphtheriae.* The 0 hr. sample was uninoculated medium. ○—○, lactic acid; ●—●, pyruvic acid.

This is further borne out by the results in Table 1 in which analyses of 48 hr. cultures are shown arranged in order of descending Lf values. It is clear from Table 1 that neither the α-ketoglutaric acid values nor the pyruvic acid values bear any relationship direct or indirect to the toxin titres. The extreme variability of the amounts of α-ketoglutaric acid that were found is an indication of the lack

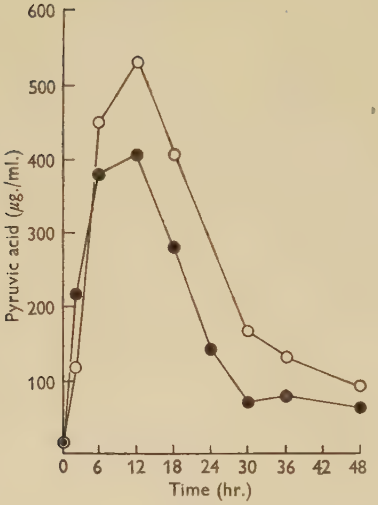

Fig. 3

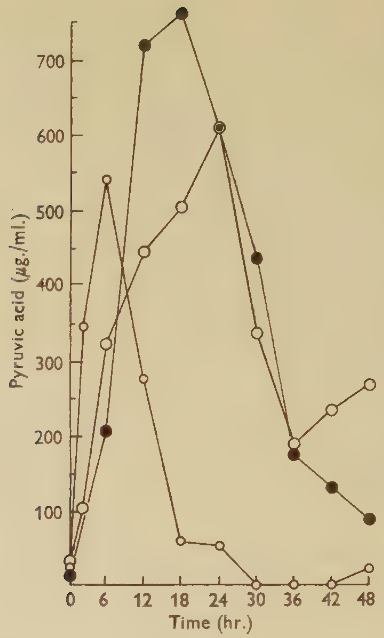

Fig. 4

Fig. 3. Pyruvic acid production by the CN 2000 strain of *Corynebacterium diphtheriae* with and without added iron. The 0 hr. sample was uninoculated medium. ○—○, no added iron; ●—●, 4 μg. Fe/ml. added.

Fig. 4. Pyruvic acid production by the G12/6 strain of *Corynebacterium diphtheriae* without added iron and by the SM1 strain with and without added iron. The 0 hr. samples were uninoculated medium. ∘—∘, G12/6 strain; ○—○, SM1 strain without added iron; ●—●, SM1 strain with 3 μg. Fe/ml. added.

Table 1. *Analyses of* 48 *hr. cultures of* Corynebacterium diphtheriae *for toxin, pyruvic acid and α-ketoglutaric acid*

Culture number	Toxin (Lf/ml.)	Pyruvic acid (μg./ml.)	α-ketoglutaric acid (μg./ml.)
SC 1257	345	90	30
1346	340	96	0
1440	328	408	8
1394	316	142	0
1391	312	98	0
1272	305	242	304
1438	296	258	0
1444	280	49	0
1442	275	78	0
1280	263	442	518
1331	262	12	0
1441	244	188	0
1393	236	187	65
1439	216	122	100
1249	202	—	646
1443	175	0	0
1389	170	158	0

of control over the organism that existed in this work although the conditions of cultivation were kept as uniform as possible. Even when the peak values for pyruvic acid (occurring after 12 hr. growth) are used there is still no apparent relationship to the final toxin yield, for in five cultures in which final (48 hr.) toxin titres of 202, 263, 305, 340, and 345 Lf/ml. were found, the 12 hr. values for pyruvic acid were 300, 710, 637, 536 and 654 μg./ml., respectively.

*Experiments with the G*12/6 *and SM*1 *strains.* Figure 4 shows the production of pyruvic acid by the G12/6 and SM1 strains. In the case of strain G12/6 the results were very similar to those found with strain CN2000 although the peak production occurred after only 6 hr. instead of 12 hr. as is usual with strain CN2000. The SM1 strain when grown without any additional iron gave a relatively slow production of pyruvic acid which continued until 24 hr. after inoculation. In the presence of iron (3μg./ml.) the toxin production was nil (compared with 100 Lf/ml. in the control) and the production of pyruvic acid rapid, reaching a peak after 12 hr. Disappearance of pyruvic acid started after 18 hr. and was rapid. Thus in the presence of iron, pyruvic acid production by strain SM1 was more like that found for strain CN2000.

DISCUSSION

In 1958 Yoneda & Ishihara concluded that iron exerted a 'distinct influence not only on the liberation of toxin but also on the pyruvate metabolism of the organism'. From the results presented in the present paper it seems clear that no such influence was exerted on pyruvate metabolism when strain CN2000 of *Corynebacterium diphtheriae* was grown in aerated submerged culture. The SM1 strain (the one used by the Japanese workers) did appear to give a change in pyruvic acid production in the presence of iron but when the toxin titres were 100 Lf/ml. for the control culture and 0 Lf/ml. for the one containing iron, the final pyruvic acid concentrations were 94 and 271 μg./ml. respectively. Yoneda & Ishihara on the other hand found that as the toxin titre fell from 60 to 0 Lf/ml. the pyruvic acid concentration fell from 220 to 11 μg./ml.; thus a serious difference exists between the two sets of results. The differences in the two methods of cultivation must be borne in mind, but regardless of this, the present results with the SM1 strain, together with the fact that the pyruvic acid metabolism of strain CN2000 was virtually unaffected by the presence of 3 μg. Fe/ml., seem to rule out the possibility that toxin and pyruvic acid production are necessarily closely linked.

The rapid decrease in lactic acid concentration and corresponding increase in pyruvic acid shown in Fig. 2 suggest the presence in *C. diphtheriae* strain CN2000 of a powerful lactic acid dehydrogenase. After incubation for 12 hr. the cultures contain *c.* 10^8–10^9 viable organisms/ml. compared with *c.* 10^{10} viable organisms/ml. after 24 hr., so that the production of the high yields of pyruvic acid has been brought about by relatively few organisms. The cause of the change from production to disappearance of pyruvic acid is obscure, but it may be that shortage of oxygen, which has already been suggested as the factor which limits the growth rate (Edwards, 1960), is responsible for the 12 hr. culture beginning to reduce some pyruvic acid to lactic acid.

I wish to thank Dr C. G. Pope for discussions during the course of this work and Miss V. Gooch and Mr H. R. Hazelton for their technical assistance.

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The Influence of Trace Elements on the Metabolism of Aromatic Compounds by Soil Fungi

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SUMMARY

The effect of copper, iron, manganese and zinc on the metabolism of catechol, *o*-, *m*- and *p*-hydroxybenzoic, *o*-, *m*- and *p*-methoxybenzoic, 3:4-dimethoxybenzoic and vanillic acids was investigated. *Aspergillus niger*, *Hormodendrum* sp. and *Penicillium* sp. mycelia which were deficient in the various trace elements were the organisms used. Only iron had any marked effect on the rates of metabolism and on the accumulation of intermediate products of metabolism in the substrate solutions. The intermediate products identified were: protocatechuic acid from *m*- and *p*-hydroxybenzoic and *p*-methoxybenzoic acids; catechol from *o*-hydroxybenzoic acid; vanillic acid from 3:4-dimethoxybenzoic acid; *o*-, *m*- and *p*-hydroxybenzoic acids from the corresponding mono-methoxybenzoic acids.

INTRODUCTION

In the present paper the investigations previously reported (Henderson & Farmer, 1955; Henderson, 1957) about the metabolism of aromatic compounds by fungi have been continued with a study of the influence of copper, iron, manganese and zinc on their metabolism. By using mycelia deficient in these elements it has been possible to obtain further information on metabolic pathways. The principal test organism was *Aspergillus niger* which has been so widely used in trace element work. It was established initially that this organism metabolizes aromatic compounds by the same pathway as previously reported for other soil fungi (Henderson & Farmer, 1955).

METHODS

Organisms. The following fungi were used: *Aspergillus niger* M (obtained from Dr E. G. Mulder, Groningen, Holland); *Hormodendrum* sp. no. 1 from sand under Scots Pine in an afforested sand dune; *Penicillium* sp. no. 13 from moorland soil. The last two organisms were used in previous investigations (Henderson & Farmer, 1955; Henderson, 1956, 1957).

Media. The basal growth medium was that of Donald, Passey & Swaby (1952) from which trace elements were removed by autoclaving with alumina and to which solutions of the required trace elements were subsequently added in the form of Matthey 'Specpure' H_3BO_3, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.5H_2O$, and $(NH_4)_6Mo_7O_{24}.4H_2O$ (Johnson, Matthey and Co. Ltd, Hatton Garden, London). This medium was modified for *Hormodendrum* sp. and *Penicillium* sp. For these, 0·5 % (w/v) of Difco vitamin-free Casamino acids was added and for the Penicillium the medium was adjusted to pH 5. Pure cane sugar was used. Pyrex flasks were used throughout and all glassware was soaked in HCl solution (*c.* 17·7 %, w/v) and then rinsed five times with double-distilled water from a glass still. The trace element deficiency of each batch of medium was tested by growing *Aspergillus niger* on it in static culture and noting the resulting growth.

Inocula. Trace element deficient spores of *Aspergillus niger* were obtained from 7-day growth on 10 ml. liquid medium ($NaNO_3$, 0·3 g.; KH_2PO_4, 0·1 g.; KCl, 0·05 g.; $MgSO_4.7H_2O$, 0·05 g.; glucose, 1 g.; water, 100 ml.), which had been treated with alumina. The growth was transferred to test tubes containing 15 ml. 1/1000 (v/v) Tween 80 solution made up with double-distilled water. On shaking the spores became detached from the hyphae and were poured off suspended in the Tween solution. Subsequent preparation of the suspensions was as described by Henderson (1956). The suspensions for static culture were adjusted to contain $3·5 \times 10^7$ spores/ml. and for shaken culture 5×10^8/ml.; 0·1 ml. suspension was added to each flask of medium. Sufficient sporing growth of *Hormodendrum* sp. and *Penicillium* sp. could not be obtained by the above method and cultures growing on cellophan strips laid on potato glucose agar were used. Spore suspensions of these two organisms were adjusted to contain 6×10^8 spores/ml.

Cultural conditions. Aspergillus niger was grown by both static and shaken culture. Fungal mats were obtained from static cultures by inoculating 25 ml. growth medium, contained in 100 ml. conical flasks, and incubating for 6 days at 26°. A thick hyphal mat, densely covered with spores, was obtained in the presence of all trace elements, while in the absence of specific elements the resulting growths were similar to those reported by Donald *et al.* (1952). Pellets were produced when similar flasks were incubated on a shaking machine (Webley & Duff, 1955) for 3 days. *Hormodendrum* sp. and *Penicillium* sp. were grown only in shaken culture and required incubation periods of 5 and 3 days, respectively.

Replacement technique. After the growth period the culture medium was decanted and the hyphal mats of *Aspergillus niger* from static culture and the pellets of the three species from shaken culture were washed three times with sterile double-distilled water before adding 25 ml. 0·01 M-substrate solution per flask. Solutions of salts of the acids under investigation were prepared at pH 6·5 and treated for removal of trace elements by autoclaving with alumina, and then sterilized by autoclaving. Catechol, which could not be autoclaved, was dissolved in double-distilled water and sterilized by filtration through a sintered glass filter which had been treated with HCl for the removal of trace elements. Mats were incubated at 26° for 1, 2, 3 and 5 days and pellets for 1, 2 and 3 days in the presence of substrate solutions. The mycelium was then filtered off and the solutions acidified and extracted with ether.

Estimation of residual substrates and intermediates. The ether extracts were evaporated to dryness and the residues dissolved in 8 drops of absolute ethanol and equal samples of the ethanolic solutions were applied to Whatman No. 1 filter paper. The papers were developed with *n*-butanol/ammonia (sp.gr. 0·880)/water (80/5/15 ml.) during 16 hr. at 21°, by the descending method. They were sprayed with diazotized sulphanilic acid (Bray, Thorpe & White, 1950), ammoniacal silver nitrate or buffer spray (Fewster & Hall, 1951) for the detection of residual substrates, and of intermediates when these were known from previous investigations (Henderson

& Farmer, 1955; Henderson, 1957). When deficiency of a particular metal was affecting metabolism, the differences in rates of metabolism were so distinct that they could readily be detected by examination of the size and intensity of spots obtained on paper chromatograms. Occasionally residual substrates were estimated spectrometrically, as was done for *o*-hydroxybenzoic acid. Following the extraction of catechol (see later) the substrate solutions were acidified and extracted with ether. The products obtained on evaporation of the ether were dissolved in 0·05 N-NaOH and the acid was estimated by ultraviolet absorption measurements at 295 mμ ($\epsilon = 3{\cdot}53 \times 10^3$).

Protocatechuic acid, when formed as an intermediate from *m*- and *p*-hydroxybenzoic acids, was estimated as follows. Products from ether extracts of substrate solutions were dissolved in a known volume of ethanol and 0·033 ml. applied to No. 54 Whatman paper by a micropipette. The papers were developed with propanol saturated with borax buffer (pH 8) for 7 hr., dried, then further developed with butanol saturated with borax buffer (pH 8) for 24 hr. to obtain good separation of residual hydroxybenzoic acid from protocatechuic acid. The position of protocatechuic acid was located by its fluorescence in ultraviolet radiation and the strip of paper containing the acid cut out. The acid was extracted by immersing the paper in 5 ml. borax buffer (pH 8) held at 50° for 15 min. Protocatechuic acid was estimated by ultraviolet absorption measurements at 293 mμ, where the acid has an ϵ maximum of *c*. $6{\cdot}3 \times 10^3$.

Catechol was estimated by taking culture solutions to pH 7–8 with $NaHCO_3$, and extracting with ether to remove the catechol. After washing, the ether extracts were taken to dryness and the residues dissolved in borax buffer (pH 8). Catechol was estimated from ultraviolet absorption measurements at 280 mμ where ϵ is approximately $3{\cdot}97 \times 10^3$.

RESULTS

Influence of copper, iron, manganese and zinc on the metabolism of o-, m-, *and* p-*hydroxybenzoic*, o-, m- *and* p-*methoxybenzoic*, 3:4-*dimethoxybenzoic and vanillic acids and catechol by* Aspergillus niger

Only iron-deficient mats showed a rate of metabolism which was consistently slower on all substrates than was that of the control. Under non-deficient mats the substrates had usually disappeared by 3 days, while under iron-deficient mats substrates were still present after 5 days, frequently in large amounts. This may be partly attributed to the decreased amount of growth in the iron-deficient cultures. However, the growth yields in zinc-deficient cultures were similar to those of iron-deficient cultures, yet there was not the consistent markedly decreased rate of metabolism. This points to the participation of iron in the initial stages of metabolism. The amounts of growth were not so markedly decreased in the absence of copper and manganese, although the types of growth were distinct—the pale buff-coloured sporulation of copper-deficient cultures and the 'cauliflower' growth, with almost no sporulation, of manganese-deficient cultures. Copper and manganese did not appear to influence the rates of metabolism.

The experiments with *o*-, *m*-, and *p*-hydroxybenzoic acids and catechol were repeated with pellets from shaken cultures. Metabolism of pellets was more rapid than was that of mats and the results were not so well defined. However, the same trend was obvious: metabolism by iron-deficient pellets was delayed and the disappearance of all four substrates took about twice as long as with non-deficient pellets. This is illustrated in Table 1 by figures obtained for residual *o*-hydroxybenzoic acid when it was being metabolized by non-deficient and iron-deficient pellets. Slight delays sometimes occurred with manganese and zinc deficiencies.

Table 1. *Metabolism of* o-*hydroxybenzoic acid and formation of catechol by shaken cultures of normal and iron-deficient* Aspergillus niger

Pellets of the fungus were incubated with 25 ml. 0·01 M-*o*-hydroxybenzoic acid solution at 26° for the stated number of days. Residual acid and catechol were extracted with ether from substrate solutions and estimated from ultraviolet absorption measurements at 295 and 280 mμ, respectively.

	mg. residual *o*-hydroxybenzoic acid/25 ml.		mg. catechol/25 ml.	
Days	+Fe	−Fe	+Fe	−Fe
1	8·5	17·7	5·8	2·0
2	< 0·04	12·9	< 0·02	1·8
3	< 0·04	7·2	< 0·02	1·7

Intermediate products of metabolism were formed by mats and pellets but they were present for a longer period of time and in larger amounts when produced by iron-deficient mycelia than by non-deficient mycelia or by those deficient in copper, manganese or zinc. Also the accumulation of intermediates was less distinct when they were formed by pellets than by mats. The intermediates were: catechol from *o*-hydroxybenzoic acid; protocatechuic acid from *m*- and *p*-hydroxybenzoic acids; vanillic acid from 3:4-dimethoxybenzoic acid; *o*-, *m*- and *p*-hydroxybenzoic acids from the corresponding monomethoxybenzoic acids and, in addition, protocatechuic acid from *p*-methoxybenzoic acid. Catechol was identified by comparison of the infrared spectra of crystalline residues, obtained from ether extracts of substrate solutions, with those of catechol. Figures for the accumulation of catechol formed from *o*-hydroxybenzoic acid by iron-deficient pellets are given in Table 1. On one occasion protocatechuic acid accumulated during the metabolism of vanillic acid. No intermediate products from catechol were detected by the techniques used. In previous work (Henderson, 1957) the hydroxybenzoic acids, protocatechuic acid and vanillic acid were shown to be intermediates when the corresponding methoxybenzoic acids, *p*-hydroxybenzoic and 3:4-dimethoxybenzoic acids, respectively, were metabolized by *Hormodendrum* sp. no. 1 and *Penicillium* sp. no. 13.

Influence of iron, manganese and zinc on the metabolism of o-, m- *and* p-*hydroxybenzoic acids and catechol by* Hormodendrum *sp. and* Penicillium *sp.*

These fungi could not be grown to form mats on the medium used for *Aspergillus niger*. However, satisfactory growth was obtained by slight modifications of this medium (see Methods) and by incubation on a shaking machine. When the growth medium was replaced by substrate solutions the rate of metabolism of substrates by iron-deficient mycelium of *Hormodendrum* sp. was approximately half of that by non-deficient mycelium. Zinc and manganese deficiencies did not alter the rate of metabolism. *Penicillium* sp. showed a slightly less-marked delay with iron-deficiency, but it also showed some delay with zinc and manganese deficiencies.

As with *Aspergillus niger*, intermediate products of metabolism were detected over a longer period of time, and in larger amounts, when formed by iron-deficient mycelia than when formed by non-deficient mycelia or those deficient in manganese or zinc. The accumulation of intermediates was, however, less evident than with *A. niger*. Paper chromatograms indicated that protocatechuic acid accumulated when it was formed from *m*- and *p*-hydroxybenzoic acids by the *Hormodendrum* sp. and *Penicillium* sp. The amounts present in substrate solutions of *Hormodendrum* sp. were estimated by ultraviolet spectrometry (Table 2). *o*-Hydroxybenzoic acid was not metabolized by *Hormodendrum* sp., while catechol formed from it accumulated in the presence of *Penicillium* sp. The formation of protocatechuic acid from *m*-hydroxybenzoic acid by *Hormodendrum* sp. was confirmed by spectrometric examination of extracts from substrate solutions. The position of the absorption maxima at 293 and 253 mμ in borate buffer was in agreement with those of a solution of authentic protocatechuic acid.

Table 2. *Accumulation of protocatechuic acid, formed as an intermediate product in the metabolism of* m- *and* p-*hydroxybenzoic acids, by shaken cultures of normal and iron-deficient* Hormodendrum *sp.*

Pellets of the fungus were incubated with 25 ml. 0·01 M-*m*- and *p*-hydroxybenzoic acid solutions at 26° for the stated number of days. Residues from ether extracts of substrate solutions were applied quantitatively to paper chromatograms. After development of the papers, protocatechuic acid was extracted and estimated by ultraviolet absorption measurements at 293 mμ.

	μg. protocatechuic acid/25 ml.			
	+Fe		−Fe	
Days	*m*-hydroxybenzoic acid	*p*-hydroxybenzoic acid	*m*-hydroxybenzoic acid	*p*-hydroxybenzoic acid
2	30	90	60	240
5	0	0	70	680
8	0	0	40	420

DISCUSSION

The principal observation arising from the results is the general requirement for iron in the metabolism of all the aromatic compounds studied. The decreased rate of metabolism of iron-deficient mycelia would lead to a decreased rate of production of intermediate products, yet the intermediates formed in substrate solutions by such mycelia were present over a longer period of time and in larger amounts than were those formed by non-deficient mycelia. This indicates that the rate of metabolism of the intermediates was also decreased. Dagley & Patel (1957) found that protocatechuic acid oxidase activity could be restored to a dialysed preparation from *Pseudomonas* sp. by the addition of ferrous ion, but not by several other ions, while Suda, Hashimoto, Matsuoka & Kamahora (1951) restored activity to a dialysed preparation of pyrocatechase by the addition of ferrous ion. Hayaishi, Katagiri & Simon (1957) purified pyrocatechase and found that it contained two atoms of iron per molecule of enzyme protein. These findings corroborate the present ones, namely, that protocatechuic acid and catechol were more slowly metabolized when formed as intermediate products of metabolism by iron-deficient mycelia than by any others. Copper, manganese and zinc did not seem to play an important role in the metabolism of the aromatic compounds examined.

In addition, evidence has been obtained for the production of protocatechuic acid as an intermediate in the metabolism of *m*-hydroxybenzoic acid, and of catechol as an intermediate of *o*-hydroxybenzoic acid by *Aspergillus niger*, *Hormodendrum* sp. and *Penicillium* sp. Walker & Evans (1952) reported that *Pseudomonas fluorescens* oxidized *m*-hydroxybenzoic acid through gentisic acid but Evans (1947) also obtained protocatechuic acid through the action of a *Vibrio* sp. The former workers also found that *P. fluorescens* grown on *o*-hydroxybenzoic acid was simultaneously adapted to metabolize catechol.

Better defined results were obtained with static mats of *Aspergillus niger* than with the shaken pellets. It is probable that metabolism is more rapid in the better-aerated shaken flasks and thus the accumulation of intermediates is less likely to occur, while the different rates of metabolism are not so readily distinguished. Also *Hormodendrum* sp. and *Penicillium* sp. did not yield as definite results as did *A. niger*, which may be due either to the fact that trace element deficient spores could not be used or to these species having a lower trace element requirement than *A. niger*. In a study of the growth yields of six fungi pathogenic to tobacco, when various trace elements were omitted, Steinberg (1956) obtained, in the majority of cases, much higher percentage yields than he did with *A. niger*, indicating that the requirements of these fungi were much lower than were those of *A. niger*. The results obtained with *Hormodendrum* sp. and *Penicillium* sp. do, however, show that for them also iron is the most important element concerned in the metabolism of the aromatic compounds studied.

I wish to thank Miss D. Brebner for valuable technical assistance and Dr V. C. Farmer and Mr J. D. Russell for carrying out the spectrochemical analyses. I am indebted to Dr E. G. Mulder for the culture of *Aspergillus niger* and to Dr D. Gross of Tate and Lyle, Ltd. for a gift of pure cane sugar. I also wish to acknowledge the helpful advice of Dr D. M. Webley.

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Isolation of a Corynebacterium and its Transitional Forms from a Case of Subacute Bacterial Endocarditis treated with Antibiotics

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SUMMARY

A bacteriological study was made of blood specimens taken repeatedly during a 2-year period from a child with subacute bacterial endocarditis who received intensive treatment with antibiotics. The conventional bacillary form of *Corynebacterium* sp. was present in the blood and bone marrow of the patient before the beginning of antibiotic therapy and on occasions when the administration of antibiotics was suspended. These were the periods when the patient showed overt symptoms of clinical illness. When antibiotic therapy was adequate to produce clinical remission of symptoms, the infecting organism was not eradicated, but persisted in the blood in a small granule-like form that could be demonstrated and cultured only by highly specialized techniques. The cultural procedures required to bring about reversion of the granule-like form to the conventional bacillary form and the morphology of the various transitional forms that the organism assumed during the reversion process are described and illustrated.

INTRODUCTION

A recurrent problem in diagnostic bacteriology is the frequency of cultures which are negative for bacteria from the blood and body fluids of patients with febrile or inflammatory processes. A second and presumably related problem is the occasional appearance of slow growing, small, pleomorphic bacteria resembling corynebacteria in these otherwise negative cultures, particularly when the specimen originates from a patient with a chronic or recurrent illness. The isolation of diphtheroids or corynebacterium-like organisms from human blood, body fluids and tissues has a long history. The varied interpretations of their significance in disease can be traced back from the discussions and references given in recent papers by Kassel (1958), Carpenter, Howard & Lehman (1956), Kassel & Rottino (1955), Fleisher (1952), and Jiménez Díaz & Arjona (1949), to the early work of Bunting & Yates (1913*a*, *b*), Billings & Rosenow (1913) and Cunningham (1917). A possible explanation for the apparently negative findings in some of these problem cases, and for the delayed appearance of the corynebacterium in others becomes available on the basis of the observations reported in this paper. We have followed for more than 2 years the case of a child with a congenital defect of the heart and intermittent fever who was diagnosed as having subacute bacterial endocarditis (SBE) on the basis of clinical findings and blood cultures positive for corynebacteria. Bacteriological studies demonstrated the continuous presence of various non-bacillary forms of the organism in the blood stream of the host during long periods of clinical remission and apparent recovery. The influence of antibiotic therapy on the morphology of the organism is described, and the relationship between the morphological form of the organism and the clinical course of the disease in the patient is discussed.

METHODS

Collection of specimens. Venous blood specimens were drawn from the arm in the usual way. The bone-marrow specimen was taken by sternal puncture. Before taking specimens meticulous care was observed in cleansing the skin at the site of the puncture with iodine and ethanol, and strictly aseptic procedures were employed during collection of the specimens.

Culture media. Each specimen was cultured on some of the following media as indicated in the text. (1) Bacto fluid thioglycollate medium (Thio) or BBL (Baltimore Biological Laboratory, Inc. Baltimore, Maryland, U.S.A.) fluid thioglycollate medium (Th-BBL) unenriched, or enriched with 20 % (v/v) normal rabbit serum. (2) BBL trypticase soy broth containing 0·1 % (v/v) agar (TSB). (3) BBL trypticase soy agar (TSA), enriched with 6 % (v/v) normal rabbit, horse, sheep or human blood. (4) Bacto veal infusion agar (VIA), enriched with 15 % (v/v) normal horse or rabbit serum, with 15–20 % (v/v) normal human serum, or with 25 % (v/v) human ascitic fluid (AscFl). (5) Bacto veal infusion broth (VIB) enriched with 25 % (v/v) human ascitic fluid. (6) Bacto Casman medium base enriched with 6 % (v/v) normal rabbit blood. (7) A mixture of equal parts of Thio (or Th-BBL) and VIB enriched with horse or human serum or human ascitic fluid to the various final concentrations indicated in the text. (8) Fluid medium prepared from Bacto PPLO agar from which the agar was removed by filtration before heating (PPLOFl). The fluid medium was enriched with 25 % (v/v) human ascitic fluid. (9) Embryonated chick eggs, between 7 and 11 days of age. (10) Chick chorioallantoic membrane (CAM) tissue cultures maintained in Hanks's balanced salt solution containing 10–15 % (v/v) normal rabbit serum.

Fermentation and biochemical tests were carried out by using the standard methods and media described in the Army Technical Manual (TM 8-227) of 1951.

The anaerobic sugar basal medium used for fermentation studies of microaerophilic cultures contained the following ingredients (g./l.): 10, trypticase; 2·5, sodium chloride; 0·5, sodium thioglycollate; 0·75, Bacto agar; 0·02, phenol red; the medium was adjusted to pH 7·6.

Sera and ascitic fluid. The sera and ascitic fluid used for enrichment of media were Seitz filtered and stored in the frozen state. After thawing they were Seitz filtered a second time, heated at 60° for 30 min., and incubated before being added to the culture media.

Egg inoculation. Embryonated chick eggs, 7–11 days old, were inoculated with 0·1 ml. samples of the patient's blood, or with 0·1 ml. suspensions of pooled minced chorioallantoic membrane (CAM) or allantoic fluid from a previous serial egg passage of the blood specimen. The inoculum was dropped on the CAM or injected into the allantoic cavity. The eggs were sacrificed between the fourth and eighth day after inoculation. Harvests were made from the CAM and allantoic fluid. Preliminary tests had shown that amniotic fluid, amniotic membrane, and yolk-sac membrane were less suitable than CAM or allantoic fluid for passage of the organism under investigation. Harvested CAM tissues were minced before culturing on media or passing to fresh eggs. CAM and allantoic fluid harvests were tested for the presence of organisms by culturing on VIA plates containing 15 % (v/v) normal rabbit serum. The plates were examined for the development of deep granular colonies or macroscopic growth.

Tissue culture. CAM from 9- to 14-day-old embryonated chick eggs was harvested aseptically, finely minced with scissors, and suspended in Hanks's balanced salt solution containing 10 % (v/v) normal rabbit serum. Antibiotics were never added to the tissue cultures. The suspension of tissue was distributed in small glass tissue-culture dishes (Warren, Wittler & Vincent, 1955) containing a round glass coverslip in the bottom. Growth occurred from the minced explants and spread in monolayers on the coverslips. The CAM tissue cultures were inoculated on the fourth to seventh day of growth with 0·1 ml. of whole blood from the patient or with 0·2 ml. of the harvest from previously infected tissue cultures when serial passage was employed.

Staining methods. Essentially the same methods as those described by Wittler, Cary & Lindberg (1956) were used. Microscopic observations were carried out on fresh specimens or cultures by using a Cooke, Troughton & Simms phase contrast microscope with a long working distance condenser and oil-immersion objective. The material was either unstained or stained lightly with methylene blue and azur stain previously dried on the coverslip according to the method of Dienes (1939, 1942). Methanol-fixed preparations stained in Giemsa's solution buffered to pH 6·8 were also studied for comparison. Gram's method was used to detect conventional bacterial forms.

Tissue cultures grown on coverslips were stained in the dishes in which they were cultivated. After harvesting the supernatant fluids, the cultures were rinsed with two changes of Hanks's balanced salt solution and fixed with absolute methanol for 5 min. after each rinse. Methanol was removed and replaced with Giemsa stain buffered to pH 6·6. After 45 min. of staining, the coverslips were rinsed in M/150 phosphate buffer at pH 6·6, dehydrated and mounted on slides.

Photography. Photomicrographs of living cultures by phase contrast were taken with a Bausch & Lomb Model L camera and a Bausch & Lomb tungsten ribbon filament illuminator. Kodak contrast process panchromatic sheet film was used with a transmission type interference filter (wavelength 550 mμ).

Controls. The inclusion of controls at each step of these experiments was deemed imperative to permit valid interpretation of the findings. For every plate, tube or bottle of culture medium inoculated with specimens or transfers of specimens, duplicate uninoculated plates, tubes or bottles of the same lot of medium were incubated along with the test cultures, opened for examination at the same time as the test cultures, and examined and transferred in exactly the same manner as the test cultures. On the rare occasions when a contaminant appeared in the control cultures, the test cultures for that particular series were discarded and the work repeated. Thus for every positive result reported in this study, it will be understood that duplicate uninoculated medium was incubated and examined by the same methods and found to be negative for any evidence of presence of organisms.

Furthermore, awareness of the possibility of encountering inclusions similar to those described by Nelson (1936), Pappenheimer, Molloy & Rose (1945), or Van Herick & Eaton (1945) in embryonated eggs led us to make a thorough search for such contaminating agents before using eggs or CAM tissue cultures for our experiments. By the use of the various cultural, microscopic and staining techniques described above, inclusion bodies could readily be demonstrated in tissue culture preparations of the CAM of eggs of certain flocks of chickens in the Washington–Maryland area (Pl. 1, fig. 1). Moreover, passage of CAM tissues infected with these inclusions through a series of CAM tissue cultures enriched with rabbit serum resulted in reversion of the agent through a series of transitional stages to a coagulase negative *Staphylococcus aureus*. Eggs from infected flocks were not used in any experiments involving the patient's specimens.

When CAM tissue cultures were prepared, a large enough pool of membranes was used to allow for large numbers of control uninoculated cultures. These were stained at various times during the experimental period. The controls were also carried through numerous serial transfers to eliminate the possibility of a delayed appearance of inclusions during passage in the presence of serum or other nutrients. Separate controls containing individual substances, such as Hanks's balanced salt solution, serum or normal human blood, and various mixtures of these substances to duplicate those used for test specimens were always included with each experiment. For all experiments involving eggs or tissue cultures from eggs, the report here of positive results for test specimens implies, therefore, that the numerous controls for that specimen were consistently negative.

RESULTS

Blood or bone marrow specimens from a child with SBE were examined bacteriologically on twenty-three separate occasions over a period of more than 2 years during which time this patient was under close clinical observation. The results of the bacteriological studies and their correlation with the clinical findings are presented in the text and in Fig. 1. The infecting agent isolated from the patient was not consistently found in the bacillary form characteristic of its species and recognizable by conventional methods. Therefore the various non-bacillary forms, as well as the methods used for their demonstration or isolation, are each described separately in the text.

Case history and clinical course

The patient, S.S., a 4½-year-old white female, was first admitted to the hospital on 5 November 1957 with a chief complaint of intermittent fever of 4 weeks duration and diminished exercise tolerance of 7 months duration. At the age of 6 months a loud precordial murmur had first been noted, and a diagnosis of congenital heart disease was made. At the time of admission to the hospital the patient was acutely ill. Her temperature was 100·6° F., and a few petechiae on the chest and upper arms were noted. There was a harsh Grade IV apical systolic murmur. The spleen was slightly enlarged. The initial white count was 15,400 and the corrected sedimentation rate was 30. No antibiotics had as yet been administered. When positive blood and bone-marrow cultures were obtained, a diagnosis of interventricular septal defect with subacute bacterial endocarditis was made.

Administration of penicillin (3,600,000 units daily) and streptomycin (500 mg. daily) was started on 7 November at which time her temperature had reached 103° F. There was considerable improvement in the patient's condition by 12 November. Her spleen was no longer palpable, the petechiae were absent, her sedimentation rate was 13 and temperature 99·8° F. Streptomycin was discontinued, but penicillin was administered until 24 November. Four to five days after discontinuing penicillin, the patient relapsed, and her temperature rose to 103°–104° F. Blood cultures were positive for corynebacteria. Penicillin therapy was reinstituted and clinical improvement followed with a decrease in temperature to 99·4° F. by 3 December. The patient remained relatively symptomless except for a low grade fever. Oxytetracycline (750 mg. daily) was administered in addition to penicillin from 10 December 1957 until 15 January 1958, and chloramphenicol (1500 mg. daily) along with penicillin was given from 15 January to 26 February. Daily penicillin was continued while the patient, who was then symptomless, was followed as an out-patient. She neglected to take penicillin for 3–4 days at the beginning of April 1958, and for a week thereafter ran a low grade fever and felt ill. The daily dosage of penicillin was decreased to 500,000 units from 25 August 1958 to the present. Although the patient has continued to appear symptomless, the bacteriological studies described below indicate that a micro-organism remains present in her blood, presumably in a latent state.

Isolation and characterization of the original bacillary form of Corynebacterium *sp.*

One bone marrow and three blood specimens were taken during the first 3 days of the patient's hospitalization. The specimens were cultured by routine bacteriological procedures in Thio and TSB media. Small white fluffy balls of growth (Pl. 1, fig. 2) appeared in both media after several days of incubation. Gram-stained smears of the balls showed small homogeneous Gram-positive rods resembling corynebacteria (Pl. 1, fig. 3).

Blood specimens (19 November and 3 December) cultured in Thio and TSB media, yielded identical small Gram-positive rods. These cultures, however, grew more slowly and required 5–8 days for the small fluffy balls of growth to become readily visible. The isolation results were confirmed by four different laboratories on quadruplicate specimens of blood drawn on 3 December. (These specimens were cultured in the respective laboratories of two of the authors, R.G.W. and W.F.M; in addition they were cultured by Lucille B. Robinson, Veterans Administration Hospital, Mt. Alto, Washington, D.C., and by Sylvia G. Cary and Martha C. Dail, Diagnostic Section of the Department of Bacteriology, WRAIR, Washington, D.C.)

A variant morphological form developed when the original TSB cultures of the blood of 5 November were transferred to blood agar plates. The variant was Gram-negative, pleomorphic and filamentous with beads and swellings among the tangled loops (Pl. 1, fig. 4). Morphologically these organisms resembled *Streptobacillus moniliformis*, but serologically they were distinct. Agglutination tests were negative (*a*) using the Gram-negative variant and standard *S. moniliformis* serum and (*b*) using a stock strain of *S. moniliformis* and the patient's serum. The variant form was unstable, and after a series of transfers on culture media reverted to small Gram-positive rods resembling corynebacteria.

The strains isolated from the bone marrow of 7 November and from the blood specimen of 19 November were tested for their pattern of biochemical reactions. Both were positive for fermentation of glucose, maltose, lactose, sucrose, salicin, and sorbitol. Both were negative for fermentation of mannitol, negative for production of indole and nitrite from nitrate, and negative for catalase. Litmus milk became acid in 3 days, then formed a clot. Colonies on sheep blood agar plates were slow-growing, small, raised, grey and non-haemolytic. The colonies were smooth at the time of primary isolation from the patient, but when the biochemical tests were conducted (2 weeks later) the colonies had become rough. The organisms were non-motile, non-spore-forming, small, Gram-positive rods, occurring in small clumps, and showing a tendency to palisade. They required blood or serum for optimal growth. The cultures were micro-aerophilic at the time of primary isolation, but grew aerobically after 2 weeks of transfer on culture media. The organism was identified as a *Corynebacterium* sp., but the species was not identical with any of the systematically defined species.

The fluorescent antibody technique was used as an additional test to differentiate the *Corynebacterium* sp. from *Listeria*. Labelled specific anti-*Listeria* serum did not fluoresce with the patient's organism.

Sensitivity tests were performed. The *Corynebacterium* sp. was sensitive to penicillin, streptomycin, oxytetracycline, chloramphenicol, novobiocin, ristocetin and gantrisin.

Isolation and characterization of various transitional forms

The blood cultures were examined for growth forms other than conventional bacilli for two reasons. As reported above, a Gram-negative filamentous variant had already been encountered during subculture of the original blood culture. In addition, there was the possibility that treatment with antibiotics might encourage the production of L-phase variants *in vivo* which might not be as sensitive as the conventional bacillus to the action of antibiotics. On seventeen occasions blood specimens examined for non-bacillary and other atypical morphological forms were positive. For convenience we shall term these non-bacillary forms 'transitional' forms and discuss the choice of this term later.

Deep granular colonies. Sixteen blood specimens (see Fig. 1) were positive for transitional forms designated as deep granular colonies. The patient's freshly drawn heparinized blood or plasma was spread on the surface of plates containing VIA enriched with 15–20 % (v/v) normal human serum or 25 % (v/v) human ascitic fluid and incubated in candle jars. Agar block preparations stained by the method of Dienes were examined daily with a phase contrast microscope. After 5–7 days of incubation many clusters of small granules were observed which appeared to grow down into the agar generally beneath the WBC layered on the surface. Many of the granules which made up these colonies appeared to be strung together and formed a continuous cone of growth that could be traced to a depth of 10–15 μ below the surface (Pl. 2, figs. 5–7). The colonies increased in diameter during the second week of incubation, but they could not be propagated by transfer to solid or fluid media. A control blood specimen taken from one of the authors, sterile normal rabbit blood, and uninoculated control plates of the media did not produce this growth form.

The granular form appeared to remain viable when passed serially in embryonated chick eggs (7–11 days old); the patient's blood was inoculated into the allantoic sac. At each transfer the allantoic fluid and chorioallantoic membrane were cultured on plates of VIA and rabbit serum. Generally by the third serial egg passage the CAM yielded deep colonies of small granules on the plate cultures.

'Fried egg' type of colony. On three occasions (14 November 1957, 17 December 1957, and 21 January 1960) the blood cultures yielded colonies with dense granular centres growing down into the agar and lacy vacuolated peripheries composed primarily of small, medium or large round bodies. Some of the bodies were filled with granules, while others appeared as empty vacuoles. This growth form bore a striking resemblance to the so-called 'fried-egg' type of colony that is characteristic of L-phase growth. The colonies averaged 60–100 μ in diameter, although many exceeded these limits. On the first two occasions the colonies developed on plates inoculated with 3-day-old broth cultures (VIB + Thio + serum + penicillinase) of the patient's blood. On the third occasion they grew on plates (VIA + AscFl) inoculated directly with the patient's plasma. Plate 2, fig. 8, shows the appearance of these colonies in an impression preparation stained with Giemsa solution. It was common to note that the central granular core which grew down into the agar adhered to the agar rather than to the slide when impression preparations were made. Thus stained preparations exhibited an empty hole in the centre of the colonies, whereas phase contrast examination of the agar block revealed the granular cores as well as the peripheral portions of the colonies.

The 'fried-egg' colonies were culturally delicate and unstable. They could not be maintained by serial transfer. The isolate of 17 December 1957 was slightly more stable than the other two and was successfully passed through a series of three transfers on agar before being lost. An effort was made to determine the antibiotic sensitivity of this growth form at the second transfer by using the impregnated paper disk method. No valid interpretation of the results could be obtained, however, because of the slow and scanty growth.

Intracellular inclusions. During the early course of the disease when *Corynebacterium* sp. was readily isolated from the blood by routine methods, it was common to find in the large mononucleated cells of the patient's blood, and sometimes in the polymorphonuclear neutrophils, many small round cytoplasmic inclusions. These inclusions were 0·7–1·4 μ in diameter. Some cells had none or only a very few inclusions, but others had as many as 50–100 packed in the cytoplasm (Pl. 2, fig. 9). They were best observed by spreading freshly drawn blood or the buffy coat from plasma on agar plates. Agar block preparations stained with methylene blue + azur according to the method of Dienes were made immediately from the plates and examined by phase contrast. The inclusions appeared quite distinct from the normal types of granulation found in WBC. They were very difficult to distinguish in Giemsa-stained preparations. Their nature is not known. They may have been a type of non-specific 'toxic' granulation. Nevertheless, more thorough investigation of these forms is indicated for the following reasons. It was observed that the number of deep granular colonies which developed beneath WBC on agar plates was particularly high when the blood specimen contained many cells which showed cytoplasmic inclusions. Secondly, during previous investigations (Wittler, 1952; Wittler *et al.* 1956) many observations had been made on the appearance of bacilli, such as *Haemophilus pertussis* or *Corynebacterium* sp. in the cytoplasm of tissue cells. When the bacilli changed from rod-shaped to small round Gram-negative forms, their appearance and arrangement in the cytoplasm were so similar to the inclusions observed in the present studies, that the resemblance could not be overlooked. The possibility exists, therefore, that the cytoplasmic inclusions found in the patient's blood represented non-bacillary forms of the *Corynebacterium* sp.

A second type of cellular inclusion was revealed by tissue culture studies. The blood specimen collected on 11 April 1958 was frozen for 2 months until suitable tissue cultures were available. The cultures were prepared from CAM + Hanks's balanced salt solution + normal rabbit serum. The patient's blood was thawed, inoculated, and passed serially for four transfers in the tissue cultures. At the time of each harvest (on the sixth day following inoculation), the tissue cells were stained according to the method described earlier with Giemsa stain and examined for inclusions. The stained cells from the third and fourth passage showed a heavy infestation of small inclusions (Pl. 2, fig. 10). The inclusions were found in clusters around the edges of the cells and were estimated to be *c.* 0·25–0·75 μ in diameter. Since the original inoculum was diluted by a factor of 1/20,000 by the fourth serial passage, multiplication rather than mere survival of the organisms seemed probable. The absence of detectable cytoplasmic inclusions until after the sixth day of the third passage suggested, moreover, that considerable time for growth of the infectious agent was required before it reached a size visible in Giemsa-stained preparations.

Round bodies and pleomorphic forms from broth cultures. The various transitional forms that appeared in broth cultures were studied on ten occasions. During the first year of the study cultural methods for the propagation of the round forms in broth and microscopic methods for detailed morphological information were not sufficiently developed to yield complete results. For instance, the earliest blood specimens were cultured in VIB + Thio + horse serum. It was soon found that human serum gave better growth than horse serum, but the results varied, depending on the source of human serum. The final modification which gave optimal and consistent growth of the round bodies was a mixture containing VIB + Thio + 25 % (v/v) human AscFl. However, growth was not maintained for long periods in this medium. Early specimens were cultured in VIB + rabbit serum, VIB + AscFl, and Thio. Although the *Corynebacterium* sp. grew well in these media, the round bodies did not. Thio + 20 % (v/v) rabbit serum was finally found to be the most suitable medium for maintaining the round bodies in a viable state and at times for promoting reversion of these forms to corynebacteria. Even in this medium the round forms could not be carried for more than about 2 weeks.

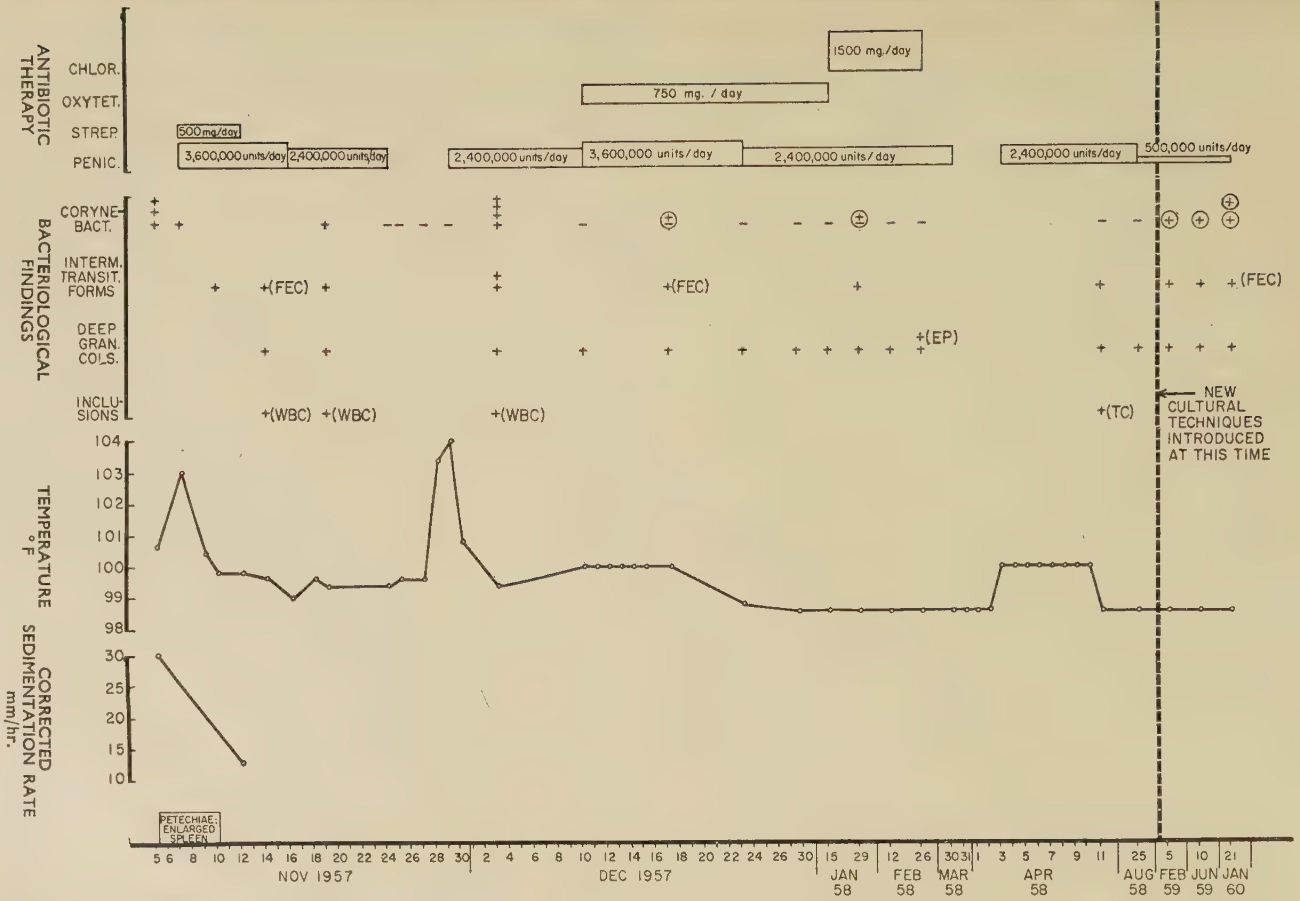

Fig. 1. Clinical and bacteriological course of a case of subacute bacterial endocarditis treated with antibiotics. (FEC) = 'fried egg' type of colonies; (WBC) = inclusions observed in white blood cells; (EP) = deep granular colonies obtained from serial egg passage of blood; (TC) = transmissible inclusions demonstrated by tissue culture passage of blood; ±⃝ = reverted Gram-positive rods observed but not isolated and established in culture; ⊕ = reverted *Corynebacterium* sp. isolated and established in culture; + = single isolation; $\overset{+}{+}$ = two separate isolations etc.; – = no corynebacterium isolated.

The transitional growth stage that consisted predominantly of round bodies produced a faint opalescent cloudiness in broth cultures within 3 days. The cloudiness was similar to that produced by Mycoplasma organisms (pleuropneumonia-like organisms) in broth and unlike that produced by bacterial growth. At times it was so faint that it was scarcely detectable even when compared with uninoculated media. The growth was studied microscopically by placing a drop of the broth culture on a small agar block and examining it with a phase-contrast oil immersion lens. During the first few days of incubation, the organisms had a more or less spherical shape and were fairly uniform in size (*c.* 1·4–1·8 μ). They were Gram-negative, but scarcely identifiable in Gram-stained preparations. Broth cultures stained with Giemsa solution at pH 6·8 showed deep rose coloured forms *c.* 1·2–1·6 μ in diameter. The shape of the bodies was readily distorted by the manipulative procedures involved in viewing them microscopically. Therefore it was concluded that possibly they lacked rigid cell walls.

It should be noted that on two occasions (Fig. 1; see 17 December 1957 and 29 January 1958) broth cultures that had shown only round bodies and no corynebacteria until the fifth day of incubation suddenly revealed a very few Gram-positive rods resembling corynebacteria. The rods disappeared by the end of the following day and never reappeared, nor did any contamination ever develop in these tubes. In the light of later studies described below it is now believed that this puzzling observation might signify that a few of the round bodies had reverted spontaneously to corynebacteria, but that the reverted bacilli were too unstable to survive in those particular cultures.

In the second year of this investigation cultural techniques were finally devised that allowed further development of the round bodies and promoted their reversion to bacillary form. An adaptation of the methods of Gilmore & Burnett (1959) was applied to the problem. Freshly drawn blood specimens (see Fig. 1, from February 1959 to January 1960) were inoculated into tubes containing VIB + Th(BBL) + 25 % (v/v) Asc Fl + penicillinase. Within 48 hr. the tubes showed clusters of small round bodies. The mechanism of multiplication of this form is not yet known. Possibly there can be simple binary division of the bodies. This was suggested by the frequent observation of two bodies attached by a thin protoplasmic connexion (Pl. 3, fig. 11). When many of the bodies showed a phase-dense sector adjacent to a phase-transparent sector (Pl. 3, fig. 12) and when large numbers of these clusters were present (48–96 hr.), the tubes were transferred *in toto* to flasks containing 50 ml. PPLO Fl + 17 ml. Asc Fl + penicillinase. The small round bodies multiplied for 3–4 days after transfer, then seemed to become stationary, then appeared to decrease in number. A second transfer in PPLO Fl + Asc Fl with or without penicillinase was made generally after 3–5 days of incubation. On one occasion, however, this transfer was delayed until the twentieth day of incubation and still gave results positive for reversion. The morphology of the round bodies, followed in various media and by phase contrast microscopy or by Giemsa-stained preparations, underwent a series of changes. The bodies became irregular in shape and developed small protrusions (Pl. 3, fig. 13). Extremely fine short thread-like forms extended from the bodies or were found free in the medium (Pl. 3, figs. 14, 15). Sometimes individual bodies appeared to be divided up into packets of delicate, pale pink (Giemsa-stained) round or oval granule-like forms. At other times these packets were found lying next to a group of pleomorphic bodies (Pl. 3, figs. 16, 17). The clusters of larger round bodies merged into masses consisting of granules, vacuoles and seemingly structureless amorphous material (Pl. 3, figs. 18, 19; Pl. 4, figs. 20, 21). Reorganization of structure then followed and chains of granules and delicate beaded filaments appeared in the masses (Pl. 4, figs. 22, 23). The plump, coccoid, or pleomorphic bacilli which first emerged from the masses were Gram-negative or Gram-variable (Pl. 4, figs. 24, 25). With further incubation they became Gram-positive. Transfer to VIB + rabbit serum helped to stabilize the reverted culture, and the bacilli then assumed the smaller slender rod-shape typical of this *Corynebacterium* species.

It has not yet been possible to follow and photograph one particular organism through its whole developmental cycle from granule to bacterium. The fact that the stages illustrated here were observed repeatedly and that reversion to *Corynebacterium* sp. was consistently obtained from them is strong evidence that the various transitional forms were non-bacillary variants of the conventional bacillus.

The results obtained by using the patient's blood were compared with the results obtained by using a normal control blood. At the same time that the patient's blood was being cultured, blood from one of the authors (whose skin flora included corynebacteria) was drawn and cultured in the same batches of media and by identical transfer methods. A variety of artifacts appeared during incubation, but round bodies and other transitional stages morphologically similar to those in the test cultures never developed in the control series, and no corynebacteria were isolated.

To establish the actual source of the isolated corynebacterium another type of control experiment was performed. Swabs of the skin of the patient's arm were cultured. Several species of diphtheroids were isolated. These organisms were larger bacilli which grew luxuriantly within 24 hr. in Thio and TSB and formed large white colonies on blood agar plates. No transitional stages were found in any of the cultures of the skin diphtheroids.

Characterization of the reverted Corynebacterium *strains*

The four *Corynebacterium* strains which had reverted from transitional forms were compared. These were designated no. 52, obtained from the blood specimen of 5 February 1959; no. 6-4*a* from the blood specimen of 10 June 1959; no. M-5 from the specimen of 21 January 1960, isolated in the laboratory of one of the authors (W.F.M.); no. 11-4d from a duplicate specimen of 21 January 1960 isolated in the laboratory of another of the authors (R.G.W.). The results of biochemical and cultural examination of these strains are recorded in Table 1. The results on the original bone marrow and blood isolates of 1957 are included for comparison. The biochemical tests carried out in 1960 were started on the same day and were run in duplicate for each strain. Aerobic strains were tested for fermentative ability using broth and semi-solid serum-enriched trypticase basal medium. Micro-aerophilic strains were tested in serum-enriched trypticase + thioglycollate basal medium and in serum-enriched trypticase semi-solid basal medium.

Recognition of the remarkable variability of these *Corynebacterium* strains under laboratory conditions is essential to any interpretation of the biochemical and cultural results. For instance, no. 52 at the time of reversion to bacillary form was extremely rough. It was transferred twice weekly for 2 months in 20% (v/v) rabbit serum broth and agar. The growth in broth became smooth, but dissociation into two types of colony variant occurred on agar. Plating directly from the broth cultures revealed only one colony type; it was similar to one of the agar variants but not identical. The smooth broth variant was stored in the refrigerator and in the deep freeze. After 1 month in the refrigerator the strain became extremely rough and could not be maintained in transfer. After 1 year in the deep freeze the strain became slightly rough. It is this slightly rough variant (52–3) that is characterized in Table 1.

Table 1. *Biochemical reactions and growth characteristics of original and reverted* Corynebacterium *strains from blood or bone marrow*

Strain ...	Original B.M. and blood	Reverted 52-3	Reverted 6-4a-3	Reverted M-5	Reverted 11-4d
Date of specimen ...	7, 19 Nov. 1957	5 Feb. 1959	10 June 1959	21 Jan. 1960	21 Jan. 1960
Date of test ...	3 Dec. 1957	16 Feb. 1960	16 Feb. 1960	16 Feb. 1960	16 Feb. 1960
Glucose	+	+	+	+	+
Maltose	+	−	−	+	+
Lactose	+	−	−	±	±
Sucrose	+	−	−	+	±
Mannitol	−	−	−	+	±
Salicin	+	−	−	±	±
Mannose		+	±	+	+
Rhamnose		−	−	±	±
Dextrin		−	−	+	+
Galactose		±	±	+	+
Sorbitol	+				
Gelatin		−	+		
Litmus milk	Acid, clot	Reduced	Reduced	−	−
Urea		−	−	−	−
$NO_3' \rightarrow NO_2'$	−	−	−	+	+
Methyl red		−	−	−	−
Indole	−	−	+	+	+
Catalase	−	+	+	+	+
Motility	−	−	−	−	−
Serum broth	Very rough	Sl. rough	Rough	Smooth	Smooth
Horse, human or sheep blood agar	Small raised grey rough colonies originally smooth	Small raised grey-white colonies	Small raised grey-white colonies	Small raised grey smooth colonies	Small raised grey smooth colonies
Rabbit blood agar		Tiny opaque colonies	Tiny opaque colonies	Tiny opaque colonies	Tiny opaque colonies
Haemolysis					
Horse		$+\beta$	$+\beta$	$+\beta$	$+\beta$
Human		$+\beta$	$+\beta$	$+\beta$	$+\beta$
Sheep	−	−	−	−	−
Rabbit	−	−	−	−	−
O_2 requirement	Aerobic, originally micro-aerophilic	Aerobic	Aerobic	Micro-aerophilic	Micro-aerophilic

Strain 6-4a was also extremely rough at the time of reversion to bacillary form. Passage on agar again resulted in dissociation into two types of colony variants. Truly smooth variants from this strain have never been obtained. One of the rough variants (6-4a-3) from broth cultures is characterized in Table 1. In addition to the changes in cultural characteristics, marked differences in the biochemical characteristics of the variants occurred. Comparison of the three variants from each of the two strains are recorded in Table 2. It will be noted that the variants (52-2 and 6-4a-2) which grew as tiny colonies and required micro-aerophilic conditions reduced nitrate to nitrite; the other variants lacked this property. Furthermore, the variant (6-4a-2) which became very rough lost the ability to produce catalase. Variant 6-4a-3 produced indole when the test was performed on cultures that were first carried through 10–12 rapid transfers in serum + thioglycollate medium.

Table 2. *Biochemical reactions and growth characteristics of variants of two* Corynebacterium *strains isolated from blood*

Strain ...	52			6-4a		
Variant ...	1	2	3	1	2	3
Date of test ...	May 1959	May 1959	Feb. 1960	May 1959	May 1959	Feb. 1960
Glucose	+	+	+	−	+	+
Maltose	+	+	−	−	+	−
Lactose	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−
Mannitol	−	−	−	−	−	−
Salicin	−	−	−	−	−	−
Sorbitol	−	−		−	−	
Gelatin	−	−	−			+
Litmus milk	Sl. alk.	Sl. alk.	Reduced	−	Proteolysed	Reduced
Urea	−	−	−	−	−	−
$NO_3' \rightarrow NO_2'$	−	+	−	−	+	−
Indole	−	−	−	−	−	+
Catalase	+	+	+	+	−	+
Motility	−	−	−	−	−	−
Tellurite	Shiny black colonies	No growth		No growth	No growth	
Serum broth	Smooth	Smooth	Sl. rough	Sl. rough	Very rough	Rough to sl. rough
Sheep blood agar	Small raised grey-white colonies	Tiny translucent colonies	Small raised grey-white colonies	Small raised grey-white colonies	Tiny opaque colonies	Small raised grey-white colonies
Haemolysis (sheep blood)	−	−	−	−	−	−
O_2 requirement	Aerobic	Micro-aerophilic	Aerobic	Aerobic	Micro-aerophilic	Aerobic

In contrast to these examples of variation are the consistent results obtained on strains M-5 and 11-4d of January 1960 (Table 1). Great precautions were taken to avoid the production of variations by laboratory manipulation. The same methods for preparation of media, inoculation, transfer, and examination of cultures were followed with the duplicate specimens of January 1960, but all the procedures were carried out quite independently in two separate laboratories. The reverted *Corynebacterium* spp. were maintained by rapid transfer in serum-enriched thioglycollate medium and tested for biochemical characteristics within a week after reversion occurred. As shown in Table 1, these two strains were identical. Both M-5 and 11-4d were smooth and micro-aerophilic as were the original blood and bone marrow isolates of 1957 before they were carried in transfer. The pattern of sugar fermentations for the 1957 and 1960 isolates shows a discrepancy only in regard to mannitol. However, the results for litmus milk, nitrite from nitrate, indole and catalase are different for the 1957 and 1960 isolates.

In view of the results on the variants of strains no. 52 and no. 6-4a, which behaved quite differently with respect to litmus milk, nitrite from nitrate, indole and catalase, one cannot conclude that the 1957 and 1960 isolates were necessarily different species of corynebacteria. For instance, M-5 and 11-4d which were micro-aerophilic produced nitrite from nitrate as did the micro-aerophilic tiny variants 52-2 and 6-4*a*-2. M-5 and 11-4d produced indole as did 6-4a-3 after it was passed in serum + thioglycollate medium. The 1957 blood and bone marrow strains, tested after they became very rough, did not produce catalase, the same being true for the very rough 6-4a-2 strain. For the difference in the litmus milk reaction of the 1960 strains there is no comparable parallel among the 1959 variants.

None of the strains isolated from the blood of the patient conformed entirely to the characteristics of the species already identified systematically. Our strains are probably more closely related to *Corynebacterium acnes* than to other taxonomically defined species of *Corynebacterium*. Until further studies can be completed, it is probably best not to use a species name.

Penicillin sensitivity tests were carried out on strains M-5, 11-4d, 52-3, and 6-4a-3. M-5 and 11-4d were sensitive to 0·1 units penicillin/ml., whereas the two rougher variants, 52-3 and 6-4a-3, were sensitive to 0·4 units/ml. and 1·58 units/ml., respectively. The very rough variant 6-4a-2 was resistant to 25 units penicillin/ml. Strain 52-3 was tested against 18 other antibacterial agents and was resistant only to gantrisin.

DISCUSSION

The foregoing results point to a correlation between the morphological form of the infecting microbe and its pathogenic potential in the infected host. The evidence for this correlation rests upon the following observations. When the patient was admitted to the hospital before receiving any antibiotic therapy, she was febrile and clinically ill. At this time the corynebacterium isolated from her blood and bone marrow was in its conventional bacillary growth form and could be cultivated on routine blood culture media. When antibiotics were administered, the patient's temperature declined and her clinical condition improved. Blood cultures then yielded a small granular form and other transitional stages of the corynebacterium which bore no resemblance morphologically or culturally to the conventional bacillary form. Nevertheless, these transitional stages were capable of reversion to the bacillary form of the corynebacterium when cultivated on appropriate media. When antibiotic therapy was interrupted, the patient suffered a recurrence of fever and illness, and the bacillary form of the organism reappeared in her blood. As soon as antibiotic therapy was resumed, the patient became symptomless, the bacillary form disappeared from her blood, but the granular form persisted. During a period of nearly 2 years of daily penicillin treatment the patient had no recurrence of symptoms, although the transitional stage of the organism remained present in her blood and retained its capacity to revert in special culture media. Furthermore, the reverted corynebacterium remained a 'penicillin-sensitive' organism when tested by the conventional antibiotic sensitivity techniques. From these observations the transitional stage of the corynebacterium appeared to be the form associated with the latent stages of the infection, whereas the bacillary form appeared to be associated with the active stages of the infection.

The possibility of the survival of bacteria *in vivo* in a latent or unrecognizable form is not a new concept, but experimental evidence to support this theory has been fragmentary. Our findings offer further experimental support for a hypothesis presented by McDermott (1958) to account for certain phenomena of 'microbial persistence' during antibiotic therapy, i.e. 'that there was something different about parasites when they were actually producing disease which made them more drug-susceptible and that micro-organisms which were not drug-resistant in the usual sense were nonetheless capable of surviving drug therapy in the tissues of man, so that antimicrobial therapy was seldom predictably eradicative'. And further, 'that there is a state of drug-indifference, that it is reversible depending on environmental changes, and that such states can be induced or favoured by the nature of the environment'.

Implications of the importance of the small granular filterable phases of bacteria to latency, recurrences and the carrier state of disease are found in Klieneberger-Nobel's (1951) discussion of these forms. However, in her review clear-cut definitions distinguishing between the true L-phase and the granular or filterable stages of bacteria were not given. Since that time, the concept of the distinction between the two forms has been clarified. According to her latest suggestion (Dr E. Klieneberger-Nobel, personal communication), the L-phase is a stable growth form which no longer reverts to its original bacterial form. L-phase growth is described as a laboratory phenomenon found *in vitro* but not *in vivo* which, by appropriate cultural methods, reveals highly characteristic colonial and morphological features. On the basis of this definition the various growth phases of the organism described in the present study could not be termed L-phases of corynebacterium, since they were all highly unstable on culture media and since some of the stages were found *in vivo*. Moreover, they were morphologically diverse and included minute granular forms, small round bodies, and a wide range of non-bacillary pleomorphic forms. Nevertheless, some of the stages on culture media were temporarily capable of producing unstable colonies with deep granular centres and lacy vacuolated peripheries which resembled the L-phase of growth in their colonial and morphological characteristics. Furthermore, individual transitional stages were morphologically similar to certain stages of the L-phase of growth as illustrated and described by Klieneberger-Nobel (1949). Other stages appeared similar to the protoplasts illustrated and described by Fitz-James (1958; see his figs. 8–11) and by Spiegelman *et al.* (1958; see their figs. 3–5 and 9). All the varied stages of the organisms found both *in vitro* and *in vivo* in the present work we term 'transitional forms' to distinguish them from both the conventional bacillary form of the organism and from the stable laboratory-produced L-phase of growth. It is our opinion that the *in vivo* transitional forms reported here and shown, at least in this case, to be of significance in the course of the disease, might well be analogous to the 'filterable' or granular forms of bacteria which Klieneberger-Nobel (1951) postulated might play a role in certain recurrent or latent infections.

The observations made on the patient S.S. are not unique. Since the investigation of the first case, we have had the opportunity to study similar cases of suspected endocarditis in three male patients (one child and two adults) all of whom had received previous antibiotic treatment. Blood and bone-marrow cultures were negative by routine cultural methods in each case. By the use of our special methods, however, transitional forms of a corynebacterium were cultured from the blood and bone marrow of these three patients. Reversion of the transitional forms to the conventional bacillary form was obtained by further culture on appropriate media. It was impossible to follow the latter patients for long periods and to carry out complete cultural studies on a series of specimens taken repeatedly from each patient. Nevertheless, all of the results obtained in the subsequent cases were in accord with the findings reported for the original case.

The accumulated evidence does not support the hypothetical contention that the *Corynebacterium* sp. might have been a contaminant from the laboratory or from the skin of the patient. The organism could not have been a laboratory contaminant, because duplicate specimens cultured in different laboratories yielded the same organism. Moreover, the enormous number of controls studied during the course of this investigation showed a very low rate of contamination and never yielded transitional forms. All cultures of the patient's blood yielded transitional or bacillary forms of corynebacterium. If this organism were a laboratory contaminant, one would expect all or most of the controls likewise to be positive for such transitional or bacillary forms. There is also good evidence that the source of the organism was not the skin of the patient. If the act of piercing the skin with a hypodermic needle were sufficient to introduce a corynebacterium into the blood sample, it would be expected that the blood sample from the author who served as a control subject would likewise have yielded corynebacteria. This did not occur. Furthermore, the skin organisms from the patient were not found as transitional forms in culture, whereas the organisms cultured from the blood specimens were usually found as transitional forms. The latter would appear, therefore, to be from a source other than the skin. Finally, the patient showed acute symptoms of illness of a clinical degree when the organism was isolated in its bacillary form. She appeared symptomless when the organism was present only as transitional forms. This correlation indicates that the source of the bacilli was systemic not superficial, that they must have been isolated from the blood and not from the skin.

The negative results for isolation of corynebacteria obtained by Kassel & Rottino (1955) when they used germ-free techniques could, we believe, be explained on the basis of the cultivation methods they used. In our hands transitional stages incubated in the presence of crystal violet in PPLO broth for the first 5 days were unsuitable for further culture and reversion to corynebacteria. Therefore, if transitional forms were present in Kassel & Rottino's specimens, their methods would not have enabled them to demonstrate and promote the propagation and reversion of these forms.

The results of the study on the biochemical characteristics of the *Corynebacterium* strains showed that the strains were capable of marked variation. The results did not necessarily prove that the strains did not belong to the same species. Our observations accumulated during these years of study on the strains from the blood lead us to believe they are all members of the same species. We showed that variations could readily be produced by laboratory manipulation. The variations in morphology suggest that other variations may occur also *in vivo* when the host environment changes during antibiotic treatment. The demonstration of the variations among the isolates indicates, therefore, an area for further study both taxonomically and serologically, before valid criteria for establishing identity or lack of identity of *Corynebacterium* spp. are available. Quite aside from any value these studies may have in assisting in the identification of the etiological agent in any specific case, we believe their primary contribution lies in the means offered for demonstration in the host of viable agents of potential pathogenicity which until now might easily escape detection, or might be dismissed as contamination of a particular culture if the appearance of growth were long delayed.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Tissue culture of chick CAM cells showing clusters of inclusions along the periphery of the cell cytoplasm. These contaminants were present in the uninoculated eggs of various flocks from the Washington–Maryland area. Giemsa stain; × 1116.

Fig. 2. Patient's blood cultured in a bottle of TSB. The small white fluffy balls of growth appeared after several days of incubation. × 1·1.

Fig. 3. Gram-positive bacillary form of the corynebacterium isolated from the patient's blood in TSB. Gram stain; × 1333.

Fig. 4. Filamentous and pleomorphic Gram-negative forms of the corynebacterium isolated from the patient's blood after transfer to blood agar plates. Gram strain; × 1333.

PLATE 2

Fig. 5. Surface of agar plate inoculated with patient's blood showing the debris of disintegrated WBC after 5 days of incubation. The arrow indicates the area below which the deep colony of granules developed. Dienes's stain and phase contrast; × 1013.

Fig. 6. Same as Fig. 5 but with the focus adjusted slightly below the surface of the agar. The arrow indicates the upper level of the deep colony of granules. Dienes's stain and phase contrast; × 1013.

Fig. 7. Same as Fig. 5 but with the focus adjusted 8–10 μ below the surface of the agar. The arrow indicates the deep colony of granules at the level of most dense growth. Dienes's stain and phase contrast; × 1013.

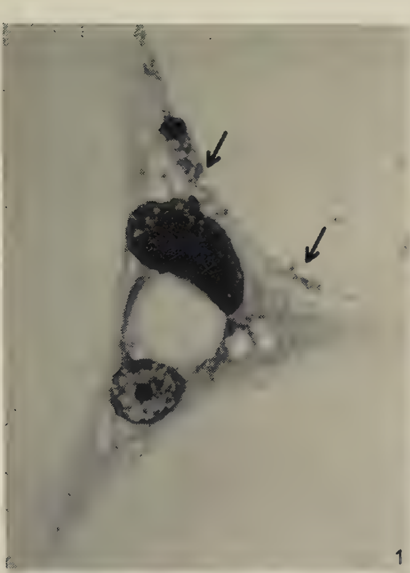
Fig. 8. 'Fried egg' type of colony showing large bodies, vacuoles and granules which made up the peripheral portion of the colony. The granular central portion of the colony remained on the agar when the impression preparation was made, thus leaving an empty hole in the centre of this specimen. Giemsa stain; × 1080.

Fig. 9. Small round inclusion bodies (indicated by arrows) in the cytoplasm of WBC of the patient's blood. In the upper left corner of the photograph is an eosinophil containing the denser and highly refractile normal cytoplasmic granulation for comparison. Dienes's stain and phase contrast; × 1013.

Fig. 10. CAM tissue culture inoculated with the patient's blood (4th serial CAM passage). Clusters of inclusions are found along the periphery of the cell cytoplasm. Giemsa stain; × 1230.

PLATE 3

Fig. 11. A chain of three round bodies. Two are still connected by thin protoplasmic material. Several bodies show differentiation of internal contents. Four-day-old culture in VIB + Th(BBL) + Asc Fl + penicillinase. Hanging drop, unstained, phase contrast; × 1029.



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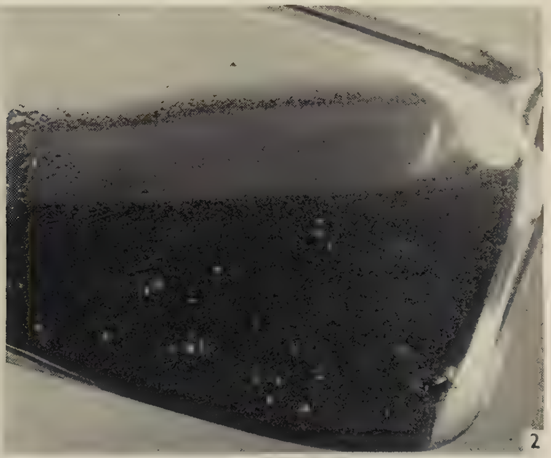

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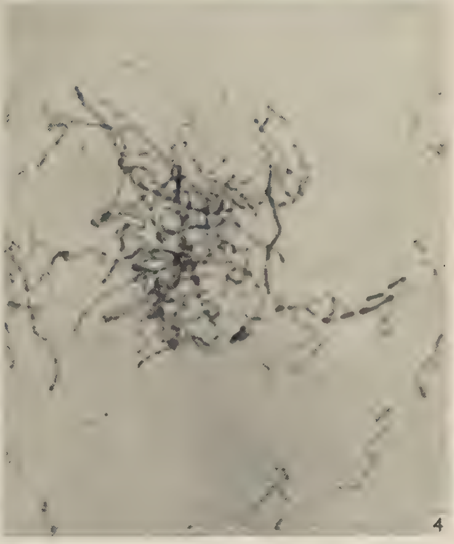

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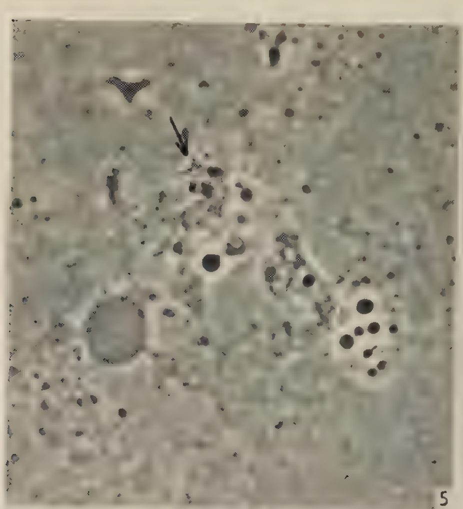
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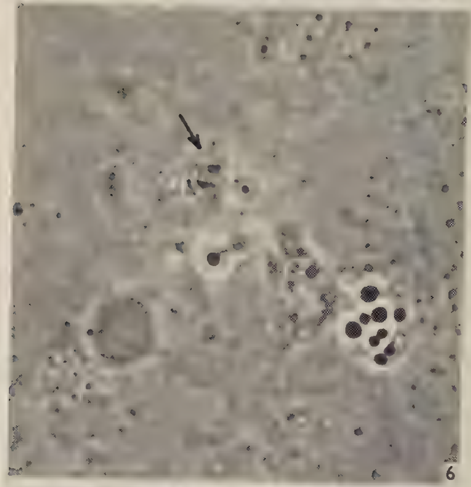
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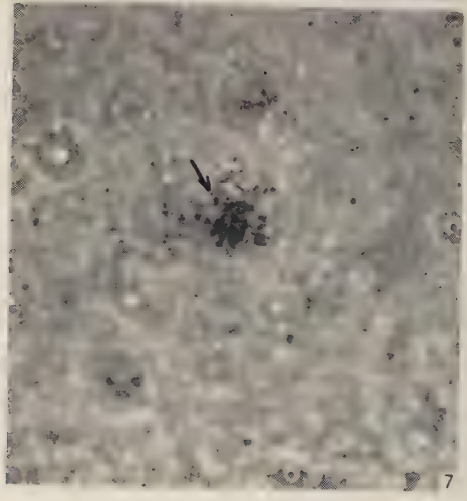
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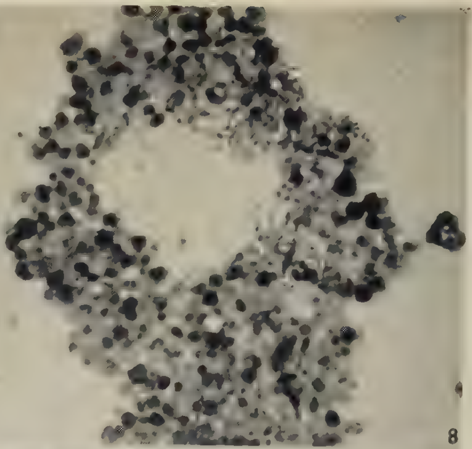
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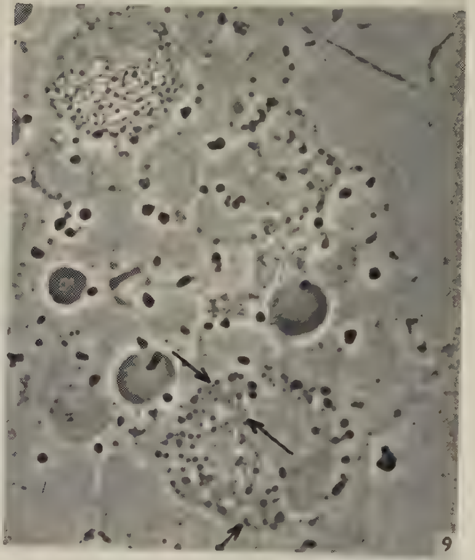
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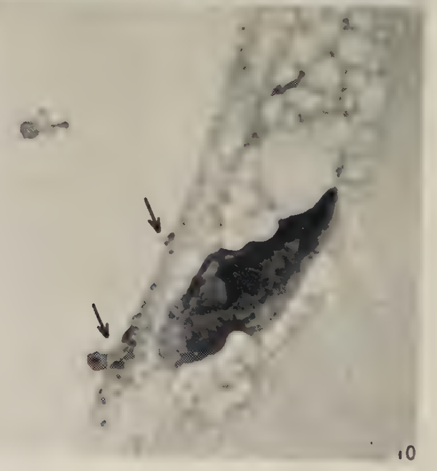
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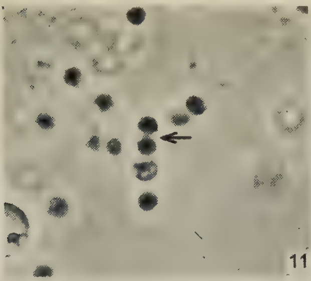
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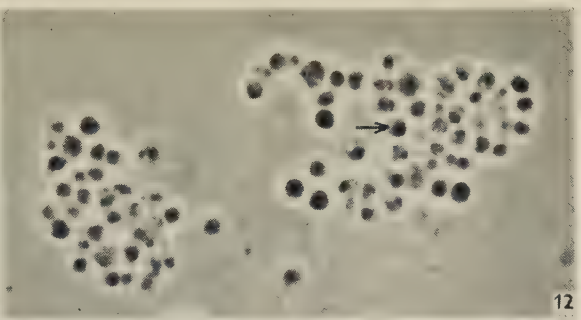
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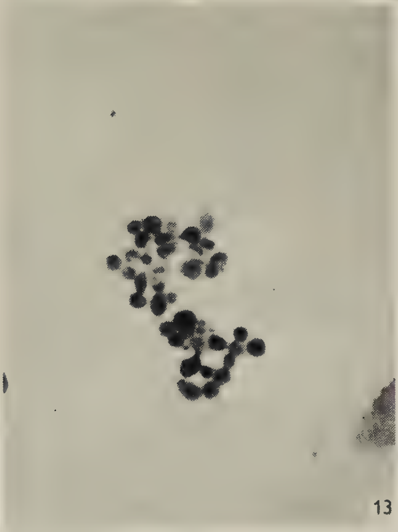
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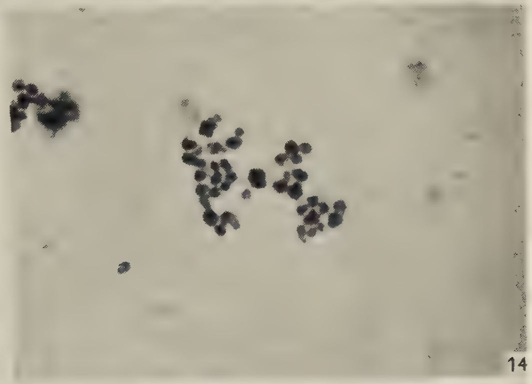
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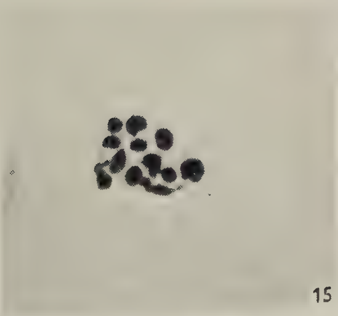
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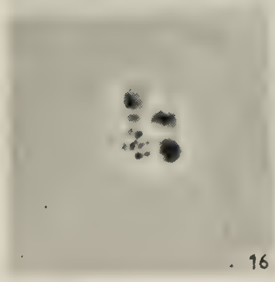
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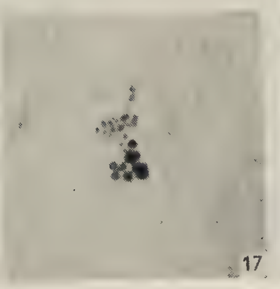
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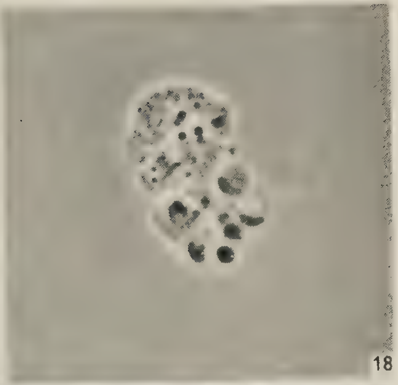
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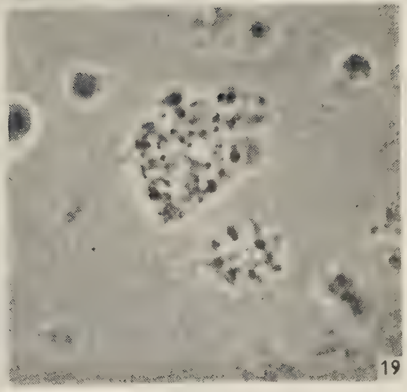
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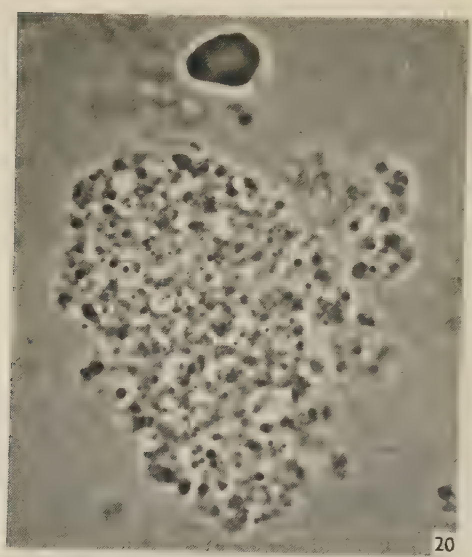
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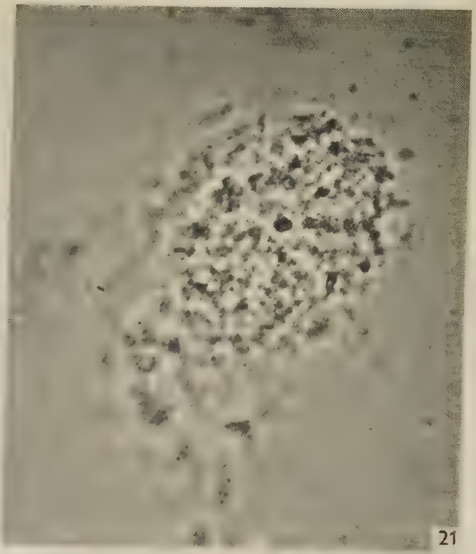
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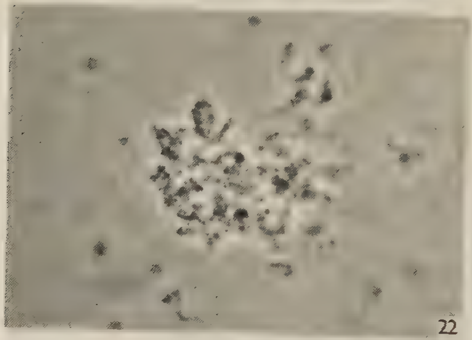
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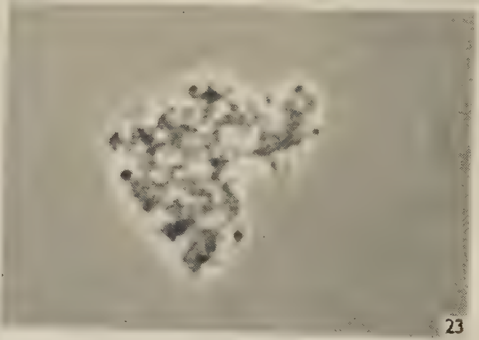
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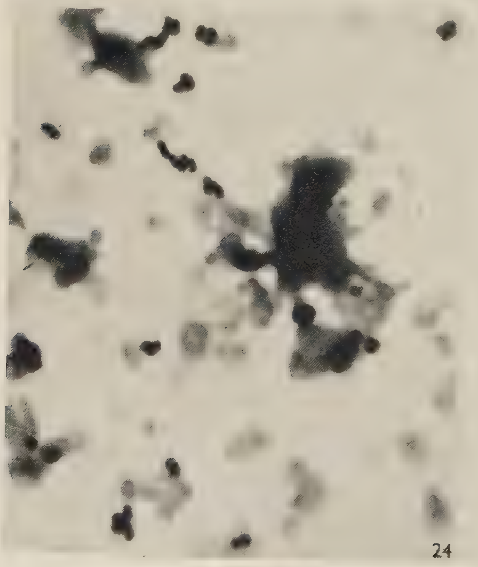
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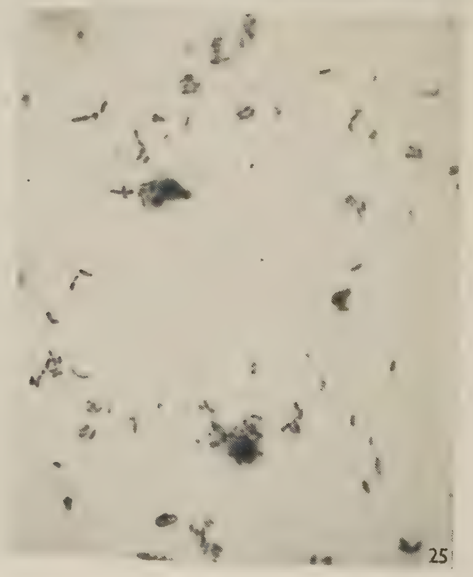
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Fig. 12. Large clusters of round bodies. Some show dark caps on a clear bubble; others show differentiation of internal contents; still others are breaking down and merging into delicate masses of amorphous material. Four-day-old culture in VIB + Th(BBL) + AscFl + penicillinase. Hanging drop, unstained, phase contrast; ×1029.

Fig. 13. Cluster of round bodies becoming irregular in shape. Several show the early stage of formation of a protrusion or small bud. Four-day growth in VIB + Th(BBL) + AscFl + penicillinase. Giemsa stain; ×1080.

Figs. 14, 15. Clusters of bodies showing fine thread-like forms extending from the bodies. Thirty-six hr. growth in PPLO Fl + AscFl. Giemsa stain; ×1080.

Fig. 16. A group of three round bodies and a cluster of granules of various sizes. Sixty hr. culture in PPLO Fl + AscFl + penicillinase. Hanging drop, unstained, phase contrast; ×1346.

Fig. 17. A cluster of delicate tiny granule-like round and oval forms lying next to a group of round bodies. Eight day growth in PPLO Fl + AscFl. Giemsa stain; ×1080.

Fig. 18. A group of round bodies in the process of breaking down and merging, and the development of an amorphous mass filled with granules of various sizes. Sixty hr. culture in PPLO Fl + AscFl + penicillinase. Hanging drop, unstained, phase contrast; × 1187.

Fig. 19. Amorphous mass filled with granules. Traces of the bubbles from the round bodies are still distinguishable. Four-day-old culture in Th(BBL) + Rabbit S. Hanging drop, unstained, phase contrast; ×1029.

PLATE 4

Fig. 20. Development of granules within a large amorphous mass composed of disrupted bodies, structureless protoplasmic material, and bubbles. Four-day-old culture in Th(BBL) + Rabbit S. Hanging drop, unstained, phase contrast; ×1029.

Fig. 21. An advanced stage of development of granules within an amorphous protoplasmic mass. The second passage of the patient's blood in PPLO Fl + AscFl was plated on VIA + AscFl and the 3-day-old growth on this plate photographed. Agar block, Dienes's stain, phase contrast; ×1029.

Fig. 22. A stage shortly before reversion to bacilli. The granules become arranged in orderly fashion and appear as beads on an extremely fine thread. Culture same as Fig. 18. Agar block, Dienes's stain, phase contrast; ×1583.

Fig. 23. The final stage before reversion to bacilli. The granules are relatively less prominent, and very delicate, filamentous bacillary forms are discernible throughout the mass. Twenty-four hr. growth of the second passage of the patient's blood in PPLO Fl + AscFl. Hanging drop, unstained, phase contrast; ×1583.

Fig. 24. *Corynebacterium* sp. (strain 6-4a) immediately after its reversion to bacillary form. Coccoid and diplococcal forms are common at this time. Twenty-four hr. growth of the fourth passage of the patient's blood in VIB + AscFl. Gram strain; ×1506.

Fig. 25. Reversion of *Corynebacterium* sp. (Strain 11-4d) from transitional form. Eleven-day growth in Th(BBL) + Rabbit S inoculated with the patient's blood. Giemsa stain; ×1080.

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The Location of the Group Antigen of Group D Streptococcus

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SUMMARY

Gel-diffusion precipitin tests showed that the Streptococcus group D antigen, as usually demonstrated by ring-precipitin tests, was serologically identical in all four species (*Streptococcus faecalis*, *S. faecium*, *S. durans*, *S. bovis*). Cell walls of representative cultures of all group D species had a similar chemical composition (determined by chromatograpy) as found by other workers. The one strain of *S. bovis* examined lacked galactosamine which was present in the cell walls of the other group D species. The group D specific antigen was found in the residue (cell contents) after the cell-wall fraction had been removed, unlike the group A specific antigen which forms an integral part of the cell wall.

INTRODUCTION

As part of a study of serological group D Streptococcus we decided to investigate the nature and location of the group D antigen; this has been little studied. By analogy with groups A, B, and C the group D substance has been assumed to be carbohydrate, but this is by no means certain. Foley & Wheeler (1945) examined four strains of group D Streptococcus of different serological types; they reported that in three strains the group specific antigen was probably protein and in the fourth strain probably carbohydrate. That the group D antigen is different in nature from the A, B, and C group antigens is also suggested by the work of Maxted (1948) and Swan (1954), who reported that the group antigen of group D streptococci was not as easily released by the action of a streptomyces enzyme as was the group antigen of group A, B and C streptococci. Technical difficulties not generally met in other groups of Streptococcus are often encountered in preparing potent group D antisera and in the extraction of the group antigen for serological identification of isolates (Shattock, 1949). Several workers have reported strains related physiologically to group D strains which were difficult to classify serologically as group D organisms (e.g. MacPherson, 1953; Higginbottom & Wheater, 1954; Perry, Wilson, Newland & Briggs, 1955). To help elucidate these problems we have examined the serological identity of the group antigens derived from representative strains of the four species of Streptococcus within serological groups D (Shattock, 1949, 1955) and have attempted to determine the location of the group antigen in the organism.

McCarty (1952*a*, *b*) showed that the group antigen of group A streptococci was located in the cell wall and was composed of rhamnose and glucosamine. Cummins & Harris (1956) when analysing the cell walls of various Gram-positive bacteria found that the composition of the cell walls of two cultures of group D strepto cocci was as follows. With *Streptococcus faecalis* 6782 the cell walls contained rhamnose, galactose, glucose, mannose, glucosamine, galactosamine, unknown hexosamine (identified by Strange, 1956, as muramic acid), alanine, glutamic acid, lysine; with *S. faecalis* 370 the cell walls contained all these compounds except galactose. These results confirmed earlier work of Salton (1952) with the cell wall of *S. faecalis* 6782.

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Since Cummins & Harris (1956) found that the cell walls of the two strains of *Streptococcus faecalis* which they investigated differed from each other chemically as much as either of them differed from that of cell walls of members of other serological groups of Streptococcus, and since the group antigen of group A Streptococcus is situated in the cell-wall (McCarty, 1952*a*, *b*) we decided to attempt to locate the group D specific substance by fractionating the cocci. We thought that chemical differences in cell-wall composition might be associated with serological differences which might account for the known difficulty in grouping some streptococci which physiologically resemble group D Streptococcus. Since the work reported in this paper was completed, Elliott (1959) recorded that he found the group D antigen in the contents of the streptococcal cell and not in the cell wall.

METHODS

Organisms and media. The organisms investigated included representatives of all species within serological group D (*Streptococcus faecalis*, and its varieties; *S. faecium*; *S. durans*; *S. bovis*; Shattock, 1955). These had been isolated from different sources (see Table 1).

Table 1. *Strains of serological group D Streptococcus used*

Species	Code	Source
Streptococcus faecalis and varieties	NCTC 6782, 370, 775	
	Magill	Human faeces
	N37 (Type 1), S161 (Type 3)	—
	GB122 (Type 4), N83 (Type 5)	Infant faeces*
	N8 (Type 8)	—
S. faecium	HGH13, HGH511	Canned ham
	P5	Pig intestine
	S748	Endocarditis
	2678	Gruyère cheese
	2MEC, 4MEC	River water
	60	Silage
S. durans	N16 (Type 11)	Infant faeces*
	Arg117, 98D, Cu2	Dried milk
S. bovis	Pearl 11, Campion	Cow dung
	BSO7, L23	Bovine rumen
	S182 (Type 2), S194 (Type 12)	Infant faeces*

* Sharpe & Shattock (1952)

Stock cultures were maintained in glucose yeast-extract litmus milk medium containing chalk, stored in the cold (4°) and subcultured every 3 months. All cultures were also preserved by freeze-drying.

The nutrient medium used throughout was glucose Lemco broth (%, w/v: Evans's peptone, 1; Lab. Lemco, 1; NaCl (Analar), 0·5; glucose, 0·5).

Serological methods

Preparation of group-specific antisera in rabbits. Cultures for vaccines were grown for 24 hr. at 37° in glucose Lemco broth, the organisms collected by centrifugation, washed twice in NaCl (0·85 %, w/v) and resuspended in NaCl (0·85%) to give an optical density equivalent to Brown's tube No. 10 (Burroughs Wellcome and Co. Ltd., Euston Road, London, N.W. 1). Samples of suspensions (6 ml./cup) were shaken with 4 g. ballotini beads (Chance No. 12) in a tissue disintegrator (Mickle, 1948; frequency 50/sec. amplitude *c.* ½ in.) for approximately 1 hr. (the rubber stoppers of the cup were covered with cellophan). The total material derived from the disintegrated cocci was used for injection. Vaccines were prepared freshly for each injection and were given at 3- to 4-day intervals, starting with two doses of 0·5 ml. followed by 1 ml. doses. The number of injections required to produce a potent group antiserum varied greatly from rabbit to rabbit. Each batch of antiserum was tested for potency (approximate reaction-time) by the ring-precipitin test against extracts from a selection of group D streptococci of different serological and biochemical types. A serum which gave well-defined rings within 5 min. was considered satisfactory. Each batch of serum was also tested for group-specificity against extracts of organisms of serological groups A to P.

Preparation of antigen extracts. Two methods were used. (1) Extraction with HCl (Lancefield, 1933) as followed by Shattock (1949). (2) Extraction with formamide (Fuller, 1938).

Ring-precipitin tests in liquid media. Tubes with an internal diameter of 2–3 mm. were used; samples of sera were dispensed first into the tubes, antigen extracts were layered on top of the sera; the reaction was allowed to develop at room temperature and the junction of serum and extract was examined for ring formation in diffuse daylight, or against a black background with a standard diffused light.

Gel-diffusion tests. Mansi's (1957) modification of the double gel-diffusion method of Ouchterlony (1953) was used. The antiserum (*c.* 0·1 ml.) was contained in a centre well and antigen extracts (*c.* 0·1 ml.) were dispensed in six wells arranged equidistantly in a hexagon around the well containing antiserum; the reaction was allowed to proceed at room temperature, and plates were inspected daily for 10 days. Lines developed usually within 2–3 days.

Analysis of cell walls

Preparation of cell-wall suspensions. The method of Cummins & Harris (1956) was used except that trypsin (Allen & Hanbury) was used as enzyme; this contained some ribonuclease (personal communication from Dr C. S. Cummins).

Hydrolysis. Cell-wall preparations from cocci harvested from 500 ml. glucose Lemco broth cultures were used for chromatographic analysis, two-thirds being taken for sugars and hexosamines and one-third for amino acids. For sugars and hexosamines, samples were hydrolysed with $2\,\text{N-H}_2\text{SO}_4$. For amino acids samples were hydrolysed with 6 N-HCl following the methods of Cummins & Harris (1956).

Chromatography. Descending chromatograms were used. For the separation of the hexoses and pentoses the ethyl acetate + acetic acid + water (9:2:2) solvent of Jermyn & Isherwood (1949) was run on Whatman no. 1 filter paper for 20 hr. and developed with aniline phthalate. Good separation of the hexoses and pentoses was obtained.

For hexosamines Fischer & Nebel's (1956) solvent, pyridine + ethyl acetate + water + acetic acid (5:5:3:1), was used on Whatman no. 1 paper, run for 24 hr. and developed with ninhydrin (0·2 %, w/v, in acetone). A good separation of glucosamine from galactosamine was obtained. Known carbohydrates, 2 % (w/v), in aqueous solution preserved with chloroform were used as markers.

Amino acids were examined by a modification of Wolfe's (1957) method on two-dimensional chromatograms on Whatman no. 4 paper: the first solvent was *n*-butanol + methylethylketone + 17 N-ammonia + water (5:3:1:1, v/v) run for 36 hr. and dried for 4 hr. at 35°. The second solvent was *n*-butanol + acetic acid + water (60:15:25, v/v) which gives a one phase solvent (Smith, 1958). After running for 40 hr. the chromatogram was developed by dipping quickly through a solution of ninhydrin (0·2 %, w/v) in acetone (95 %, v/v) + water (5 %, v/v), the paper dried and heated at 105° for 5 min. A good separation was obtained. As markers, known amino acids were used at 0·02 M in isopropyl alcohol (10 %, v/v) in water.

RESULTS

Gel-diffusion precipitin tests

Specific group antisera were prepared by using representative cultures of all species of serological group D as immunizing strains (*Streptococcus faecalis* S. 161, *S. faecum* HGH 511, *S. durans* 98 D, *S. bovis* Pearl 11). Antigens were prepared by the HCl (Lancefield) and formamide (Fuller) extraction methods from *S. faecalis* (3 strains), *S. faecium* (3 strains), *S. durans* (2 strains), *S. bovis* (4 strains). Gel-diffusion precipitin tests were set up. The centre well contained antiserum and the antigen extracts were distributed so that adjacent wells contained extracts from different species of group D streptococci. The resulting lines of precipitate which formed as antigens and antibody diffused towards each other joined to form a continuous line, that is, showed a reaction of identity (Ouchterlony, 1953; Wilson & Pringle, 1955). This result was obtained with each of the four samples of group D antisera and showed that the preparations of group D antigen from all four species were serologically identical.

Chemical and serological investigations of cell walls

Cell walls of six cultures representative of all species of Streptococcus within serological group D, prepared by the method of Cummins & Harris (1956), were examined by paper chromatography. The results are given in Table 2; included are the results for the two strains *S. faecalis* 6782 and *S. faecalis* 370 examined by Cummins & Harris. The relative amounts of the various components were estimated by the size and intensity of the spots. The major components of the cell walls of *S. faecalis* (3 cultures), *S. faecium* (1 culture), *S. durans* (1 culture) were rhamnose, glucose, glucosamine, galactosamine, alanine, lysine, and glutamic acid (muramic acid was not tested for). Aspartic acid, serine, glycine, and proline were present

Table 2. *Chromatographic analysis of cell walls of species of Streptococcus of serological group D*

The number of + signs indicates relative abundance.

Components of cell walls	*S. faecalis*			*S. faecium*	*S. durans*	*S. bovis*
	6782	370	775	4MEC	98D	Pearl 11
Arabinose	−	−	−	−	−	−
Rhamnose	++	++	++	++	++	++
Galactose	+	−	+	−	+	+
Glucose	++	++	++	++	++	++
Mannose	+	+	+	−	−	−
Glucosamine	++	++	++	++	++	++
Galactosamine	+	+	+	+	+	−
Aspartic acid	+	+	+	+	+	+
Alanine	+++	+++	+++	+++	+++	+++
Glutamic acid	+++	+++	+++	+++	+++	+++
Glycine	+	+	+	+	+	+
Lysine	+++	+++	+++	+++	+++	+++
Proline	+	+	+	+	+	+
Serine	+	+	+	+	+	+

in small quantities. One culture of *S. bovis* showed the same pattern except that galactosamine was not detected.

Serology. From cell walls of *Streptococcus faecalis* 6782 and 370, *S. faecium* 4 MEC, *S. durans* 98D extracts (HCl, Lancefield; for amide, Fuller) were prepared and tested by the ring-precipitin test against group D antisera prepared against *S. faecalis*, S161, *S. durans* 98D, *S. faecium* HGH511 and *S. bovis* Pearl 11 and against a sample of commercial group D antiserum. The only cell-wall extracts which gave any group reactions were those prepared from *S. faecalis* 6782, where weak precipitin rings were obtained with the five group antisera (Table 3).

Table 3. *HCl (Lancefield) and formamide (Fuller) extracts of cell walls of Streptococcus group D tested against five samples of group D antisera by ring-precipitin and gel-diffusion tests*

Extracts of cell walls of	Group D antisera against immunizing strains									
	S. faecalis		*S. faecium*		*S. durans*		*S. bovis*		Commercial serum	
	L*	F†	L	F	L	F	L	F	L	F
S. faecalis 6782	±	±	±	±	±	±	±	±	±	±
370	−	−	−	−	−	−	−	−	−	−
S. faecium 4MEC	−	−	−	−	−	−	−	−	−	−
S. durans 98D	−	−	−	−	−	−	−	−	−	−

± = weak precipitin ring; − = no precipitin ring; L* = HCl (Lancefield) extracts; F† = formamide (Fuller) extracts.

At this stage in the work the method of preparing cell walls was modified by omitting treatment with pepsin. Chromatographic analyses showed no difference between the chemical composition of the cell walls prepared by digestion with trypsin, containing ribonuclease, and pepsin, and that of cell walls prepared by omitting pepsin treatment. With the same strains as in the previous experiment, together with *Streptococcus bovis* BSO7, the serological activity of cell walls prepared without pepsin treatment was tested. HCl (Lancefield) extracts of cell walls and of whole organisms were compared by ring-precipitin tests against group D antisera prepared with the immunizing strains used in the previous experiment. Similar results were obtained. HCl extracts of whole organisms all gave clear-cut group precipitin rings, while HCl extracts of the corresponding cell-wall preparations gave no group ring precipitin reactions with the single exception of *S. faecalis* 6782, which again reacted weakly with the grouping sera. The absence of group antigen from the cell-wall preparations was again confirmed by gel-diffusion tests. The group D antigen was not detected in cell walls from *S. faecalis* 6782 by gel diffusion.

The location of the group D antigen

Because of the unexpected finding that the group D antigen was apparently not situated in the cell wall the following experiments were devised to test this further. HCl extracts were prepared from cell walls of a larger number of group D strains (*Streptococcus faecalis*, 6; *S. faecium*, 6; *S. durans*, 4; *S. bovis*, 4) and compared with HCl extracts of whole organisms, twice as many organisms being used to prepare the cell-wall extract. (It was thought that the apparent absence of the group D substance from the cell-wall preparations first used might have been due to a loss of organisms or cell walls during the preparation of the cell-wall suspensions.) Cultures were grown in 120 ml. glucose Lemco broth, the organism centrifuged down and washed twice with distilled water. One-third of the washed organisms were extracted with HCl (Lancefield) and two-thirds were used for the preparation of cell walls. HCl extracts of cell walls and of whole organisms were compared by ring-precipitin and gel-diffusion tests against four samples of group D antisera. No group antigen was demonstrated in the preparations of cell wall from *S. faecium*, *S. durans*, *S. bovis* or 3 out of 6 strains of *S. faecalis*. With the HCl extracts of cell walls from the other three strains of *S. faecalis*, weak delayed precipitin rings were observed; these were insignificant as compared with the reactions obtained

Table 4. *A comparison of HCl (Lancefield) extracts of whole organisms and of cell walls by ring-precipitin and gel-diffusion tests with four samples of group D antisera*

Immunizing strains for the preparation of antisera were *S. faecalis*, *S. faecium*, *S. durans*, *S. bovis*

		Group D antisera	
Antigens	No. strains tested	Ring-precipitin test	Gel-diffusion test
	Whole organisms		
S. faecalis	6	+	+
S. faecium	6	+	+
S. durans	4	+	+
S. bovis	4	+	+
	Cell walls		
S. faecalis	6	–/tr	–
S. faecium	6	–/tr	–
S. durans	4	–	–
S. bovis	4	–	–

+ = heavy ring of precipitate within 5 min. or line of precipitate in gel-diffusion test.
tr = weak delayed reaction.
– = no reaction.

with HCl extracts of whole organisms which, for every strain examined, gave clearcut rapidly formed rings. Tests in gel-diffusion plates confirmed the presence of group D antigen in whole organisms and its absence from the cell-wall preparations. No lines of precipitate were given by any cell-wall extract. The results are summarized in Table 4.

The action of trypsin (Allen & Hanbury) on the group D antigen in whole organisms was examined with 20 strains (*Streptococcus faecalis*, 6; *S. faecium*, 6; *S. durans*, 4; *S. bovis*, 4). Whole organisms treated with trypsin (as in preparation of cell walls) were extracted with HCl and the extracts compared with HCl extracts of whole untreated organisms by the ring-precipitin test against a group D antiserum. HCl extracts of whole organisms and of trypsin-treated organisms gave precipitin rings of similar intensity. HCl extracts from 9 of these strains were also compared by gel-diffusion tests, with similar results. Therefore treatment with trypsin did not destroy or release group D antigen from intact organisms.

Table 5. *Comparison of HCl (Lancefield) extracts of whole organisms and HCl (Lancefield) extracts of cell walls and cell contents against four samples of group D antisera by ring-precipitin and gel-diffusion tests*

Immunizing strains of the preparation of antisera were *S. faecalis*, *S. faecium*, *S. durans*, *S. bovis*.

Antigens	No. of strains tested	Group D antisera: Ring-precipitin test	Group D antisera: Gel-diffusion test
	Whole organisms		
S. faecalis	6	++	+
S. faecium	6	++	+
S. durans	4	++	+
S. bovis	2	+	+
	Cell contents		
S. faecalis	6	+++	+
S. faecium	6	+++	+
S. durans	4	+++	+
S. bovis	2	+	+
	Cell walls		
S. faecalis	6	–/tr	–
S. faecium	6	–/tr	–
S. durans	4	–	–
S. bovis	2	–	–

+++, ++, + = relative intensity of reaction in ring-precipitin tests.
tr = delayed weak reaction. – = no reaction.

Since the group antigen did not appear to be present in the cell walls of group D streptococci, we examined the supernatant fluid from disintegrated organisms which had hitherto been discarded after centrifuging down the cell-wall fraction. This supernatant fluid will be referred to as the 'cell contents'. The cell contents of organisms from 18 cultures were tested against four samples of group D antisera by ring-precipitin and gel-diffusion tests. A comparison was also made with HCl extracts of whole organisms and HCl extracts of cell walls from samples of the same batches of organisms as used for preparing cell contents. With all the strains of group D streptococci tested the group antigen was found in the cell contents (Table 5). The group D antigen in the preparations of cell contents was shown by gel-diffusion tests to give a reaction of identity with the group antigen extracted by HCl from the whole organism when tested against group D antisera.

DISCUSSION

In strains of Streptococcus of serological group A the group specific antigen is a polysaccharide which forms an integral part of the cell wall. It now appears that in contrast to this the group D specific antigen is in the residue after the cell wall has been removed; these results agree with Elliott's (1959) observations. He recorded that for five group D cultures of Streptococcus of different serological types, the cell walls contained type-specific antigen and the group-specific antigen was found in the cell contents and not in the cell walls. Glucose was the only sugar detected by paper chromatography in the group-specific antigen obtained from the cell contents of a strain of *S. durans*. In passing we note that a preliminary chromatographic analysis of the crude group D antigen precipitated by acetone from the cell contents of our strain of *S. durans* 98D showed the presence of glucose and ribose, the latter possibly from contaminating nucleic acid.

From the results of our experiments reported here it seems that the specific group D antigen is located in the cytoplasm of group D Streptococcus. The possibility is not yet ruled out that it may be loosely attached to the cell wall and hence separated by the method of disintegration. But with every strain of group D Streptococcus examined the cell contents contained amounts of specific group D antigen at least as much as that obtained in extracts from comparable numbers of whole organisms, as estimated by the intensity of ring-precipitin reactions. A few samples of cell walls showed a slight serological group D activity by the ring-precipitin test but not by gel-diffusion tests. It seems likely that these cell-wall preparations were slightly contaminated with the group specific antigen from the cell contents. The group D specific antigen does not appear to form an integral part of the cell wall, as does the group A polysaccharide. The presence of the group D antigen in cell contents from which the cell-wall fraction has been removed helps to explain Shattock's (1949) observation that group D antisera are more readily prepared in rabbits by using as vaccine disintegrated rather than whole organisms. It is also consistent with our experience that nutritional conditions play an important role in the synthesis of group D substance as distinct from their effect on multiplication. A medium which supports good growth does not necessarily result in good quantitative production of group D antigen.

We are indebted to Dr Ella M. Barnes, Dr C. A. E. Briggs, Dr A. E. Oxford and Dr M. Elisabeth Sharpe for providing cultures. A grant from the Agricultural Research Council in support of this work is gratefully acknowledged.

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Studies on the Mode of Action of Antifungal Heptaene Antibiotics

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SUMMARY

The effect of heptamycin (an antifungal heptaene antibiotic closely related to the ascosin–candicidin–trichomycin group) on the respiration of *Candida albicans* was studied. Heptamycin inhibited the oxidation of pyruvate, lactate and trehalose to a considerable degree. The oxidation of glucose, acetate, acetaldehyde and ethanol was only slightly affected by heptamycin. Anaerobic glycolysis was significantly inhibited. No inhibition of cell-free preparations of trehalase (obtained from *C. albicans*) or pyruvic carboxylase (from *C. albicans* and *Saccharomyces carlsbergensis*) was observed; CO_2 output by cell-free preparations was not affected either. The uptake of pyruvate and trehalose by *C. albicans* cells is apparently due to an active and inducible mechanism; heptamycin inhibits oxidation of these substances by blocking their penetration into the yeast cell. The oxidation of trehalose in induced organisms was not inhibited by heptamycin whereas that of pyruvate was. Heptamycin, candicidin B, ascosin, trichomycin and amphotericin B inhibited phosphate uptake by non-proliferating suspensions of *C. albicans*. The inhibition was observed at fungistatic concentrations (0·5–5 μg./ml.) and without any preincubation with the antibiotic. Phosphate uptake by *Debaryomyces nicotianae*, *Pichia farinosa*, *Cryptococcus albidus* and *Saccharomyces cerevisiae* was similarly affected by heptamycin. Esterification of inorganic phosphate by cell-free extracts of *C. albicans* was not affected by heptamycin. It is suggested that the fungistatic effect of heptaene antibiotics upon sensitive yeasts is mainly due to the prevention of phosphate uptake.

INTRODUCTION

The mode of action of antifungal polyene antibiotics was studied by several workers. Hickey (1953) reported that the activity of ascosin was strongly antagonized by long chain fatty acids and their derivatives. Dimmling (1955) found that trichomycin partially inhibited oxidation of glucose in various Candida strains. According to Aizawa (1956) trichomycin inhibited the induction of mannose and galactose in *Candida albicans*. Lampen, Morgan & Slocum (1957) reported that nystatin strongly inhibited both aerobic and anaerobic utilization of glucose by *Saccharomyces cerevisiae*, *C. albicans* and *Penicillium chrysogenum*. Penetration of glucose was not inhibited by the antibiotic. According to Bradley (1958*a*, *b*) inhibition by nystatin was also obtained with cell-free preparations of *C. stellatoidea*.

In the following experiments, the effects of heptamycin (Henis, Grossowicz & Aschner, 1957) and other heptaenes upon metabolism of various yeasts is described.

METHODS

Organisms: Candida albicans strains no. 26 and 27, *Debaryomyces nicotianae* no. 77, *Saccharomyces cervisiae* no. 5, *S. carlsbergensis* no. 7, *Cryptococcus albidus* no. 59, *Pichia fermentans* no. 137, obtained from the culture collection of Professor M. Aschner of the Technion, Israel Institute for Technology, Haifa, were used in this study.

Growth medium. For the preparation of cell-free extracts, the yeasts were grown on a rich medium composed of (%, w/v): glucose, 2; Bacto peptone (Difco), 0·5; Bacto yeast extract (Difco), 0·5; agar, 2; the various ingredients were taken up in distilled water; the final pH was 6·6–6·8.

Chemically defined medium for experiments with intact organisms. The following growth medium was used (%, w/v): glucose, 1; $(NH_4)_2SO_4$, 0·2; K_2HPO_4, 0·1; $MgSO_4.7H_2O$, 0·02; agar, 2, in distilled water (pH, 7·0). After sterilization of this basal medium for 15 min. at 121°, the following vitamins, sterilized by Seitz filtration, were added (in μg./ml. final concentration): thiamin, 0·1; biotin, 0·01; nicotinamide, 0·1; calcium pantothenate, 0·1; pyridoxine, 0·1; inositol, 0·5.

Assay medium was used for determination of antibiotic potency. Its composition (%, w/v) was as follows: glucose, 1; Bacto peptone (Difco), 0·5; Bacto yeast extract (Difco), 0·5; NaCl, 0·5 in distilled water; pH 7·2.

Starved Candida albicans *for measurements of respiration.* Because of the high endogenous (aerobic) respiration, *C. albicans* was subjected to the following starvation procedure: 18 hr. organisms grown on the defined medium were washed thoroughly with saline, resuspended in similar medium, but without glucose, and incubated for 8 hr. at 30° with constant shaking. The organisms were then washed again with saline; they could be kept for 3 days, provided they were stored at 2–4°.

Pyruvic acid was determined according to the method of Lu (1939).

Determinations of reducing sugar, calculated as glucose, were made by the method of Somogyi (1945) and Nelson (1944) with the Coleman Junior spectrophotometer and 19 × 150 mm. test-tubes.

Protein determinations of cell-free extracts were made by the method of Mehl (1945).

Preparations of pyruvic carboxylase and determination of activity. The methods of Green, Herbert & Subrahmanyan (1941) were followed; the Hughes press (1951) was used to obtain the crude extract. This was purified by precipitation with ammonium sulphate and the fraction precipitated between 42 and 52 % of saturation was collected. The enzymic activity/mg. protein of this fraction was seven to nine times greater than that of the crude extract. Pyruvic oxidase activity was determined manometrically by measuring the CO_2 release from pyruvate.

Determinations of phosphate were made by the method of Fiske & SubbaRow (1925).

Paper chromatography of sugar phosphates was carried out according to Burrows, Grylls & Harrison (1952) and Hanes & Isherwood (1949).

Potency of heptaenes. Antibiotic potency was tested with *Candida albicans* no. 26 and expressed as minimum inhibitory concentration (MIC). The assays (in assay medium) were run at 30° for 24 hr., the inoculum being 2×10^4 organisms/ml. final concentration. The potency assays were made just prior to the experiments described.

Heptamycin, lot no. A2, MIC 0·5 (μg./ml.); Candicidin B (Lechevalier *et al.* 1953), lot no. 244, Am 25 B, MIC 1·0 and Trichomycin (Hosoya *et al.* 1952), MIC 0·5 (kindly furnished by Dr H. Lechevalier, Institute of Microbiology, New Brunswick, N.J., U.S.A.); Ascosin (Hickey *et al.* 1952), lot no. R-16-17, MIC 6·0 (kindly supplied by Dr J. Martin and M. G. Bachman, Commercial Solvents Corp., Terre Haute, Ind., U.S.A.); Amphotericin B (Vandeputte, Wachtel & Stiller, 1955), MIC 20 (kindly provided by Dr P. Peiser, Squibb Company, Basel, Switzerland).

RESULTS

Effect of heptamycin on oxygen uptake by Candida albicans. The experiments were carried out by two different methods.

(i) Before measuring the oxygen uptake (in the Warburg apparatus) the organisms 12 mg. equiv. dry weight/ml. were preincubated for 2 hr. with 100 μg. heptamycin/ml. in phosphate buffered (M/30, pH 7·0) saline. The organisms were then washed thoroughly with saline to remove free heptamycin.

(ii) Oxygen uptake was measured without any preincubation, in the presence of heptamycin (20 μg./ml.) which was added simultaneously with the substrate. The results are summarized in Table 1 and Figs. 1 and 2.

Table 1. *Effect of preincubation with heptamycin on the oxidation of various substrates by* Candida albicans

The experiment was carried out in the Warburg apparatus. The reaction mixture contained 0·5 ml. of the substrate (0·15 M), 1 ml. of normal saline, 1 ml. phosphate buffer (0·2 M, pH 7·2) and yeast, 12 mg. dry weight equiv. Total volume 3·0 ml. In one series, the yeast suspension was preincubated for 2 hr. at 30° with heptamycin (100 μg./ml.). The antibiotic was removed by centrifugation, and the cells were resuspended in the reaction mixture. The control series was incubated in saline phosphate buffer without heptamycin. Cell suspensions of both series were then transferred to Warburg vessels and oxygen uptake measured as outlined.

The substrate was placed in the side arm and transferred to the main compartment after an equilibration period of 15 min. The central well contained a piece of filter paper saturated with 0·2 ml. of 15 % (w/v) KOH. Gas phase: air; bath temperature: 30°. Oxygen uptake measured for 2 hr.

Substrate	Preincubation with heptamycin	Oxygen uptake μl. O_2/hr./12 mg. yeast		Inhibition (%)
		Total	Net	
None	–	99	–	—
	+	33	—	66·7
Glucose	–	782	682	—
	+	605	572	16·3
Lactate	–	350	251	—
	+	122	89	64·6
Pyruvate	–	466	367	—
	+	72	39	89·4
Acetate	–	654	555	—
	+	518	485	12·7
Ethanol	–	719	620	—
	+	513	480	22·6
Acetaldehyde	–	562	463	—
	+	439	406	12·4

Heptamycin inhibited the oxygen uptake with sodium pyruvate as substrate. The inhibition was only slight when pyruvate was replaced by glucose, sodium acetate, acetaldehyde or ethanol. Similar results were obtained whether or not the organisms were preincubated with heptamycin. A lag of about 60 min. was noted in the oxygen uptake with sodium pyruvate as a substrate. However, this lag period was significantly decreased when organisms were preinduced with pyruvate. It may thus be assumed that the lag period was due to a diminished permeability of pyruvate in the uninduced organisms. However, inhibition of oxygen uptake by heptamycin was observed whether or not the organisms were preinduced (Fig. 2).

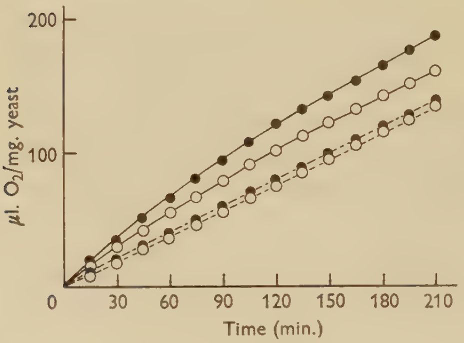

Fig. 1

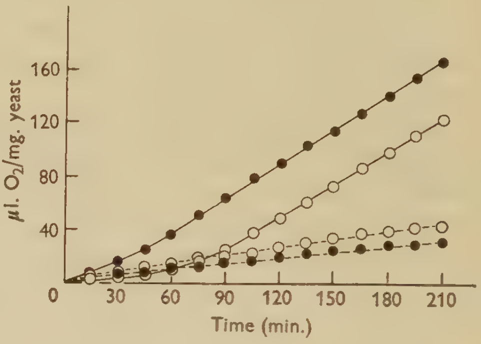

Fig. 2

Fig. 1. Effect of heptamycin on the oxidation of glucose and acetate by *Candida albicans*. The reaction mixture composition was described (Table 1). Heptamycin (60 μg.) was placed in the side arm together with the substrate and introduced to the main compartment of the Warburg vessel after an equilibration period of 15 min. ●——●, glucose; ○——○, glucose + heptamycin; ●----●, acetate; ○----○, acetate + heptamycin. The curves represent net oxygen uptake, after endogenous respiration was subtracted.

Fig. 2. Effect of heptamycin on the oxidation of pyruvate by *Candida albicans*. Organisms both induced and uninduced with pyruvate were used. Induction was carried out by incubating yeast (12 mg. dry weight equiv./ml.) with 0·025 M-sodium pyruvate and M/15 phosphate buffer (pH 7·2) for 4 hr. The organisms were then washed thoroughly and resuspended in saline. Experimental details as described (Fig. 1). *Uninduced organisms*: ○——○, pyruvate; ●----●, pyruvate + heptamycin. *Induced organisms*: ●——●, pyruvate; ○----○, pyruvate + heptamycin.

Pyruvate utilization of Candida albicans *in the presence and absence of heptamycin.* Experiments were performed in order to ascertain whether or not *C. albicans* is able to utilize pyruvate by a pathway which is not affected by heptamycin. The disappearance of pyruvate from the reaction mixture in the presence and absence of heptamycin was measured. The results are summarized in Fig. 3. It may be seen that in the absence of heptamycin full-rate consumption of pyruvate was obtained after a lag period of *c.* 1 hr.; however, no pyruvate was consumed in the presence of heptamycin. Thus, results comparable to those of oxygen uptake (Fig. 2) were obtained.

Various investigators have shown that pyruvate enters the cell by an active mechanism (Smythe, 1938; Suomalainen & Oura, 1958). Assuming that such mechanism functions in *Candida albicans* inhibition of pyruvate consumption by heptamycin may be due to the blocking of the substrate at the surface of the yeast cell, and no effect of heptamycin on pyruvate metabolism in cell-free extracts can be anticipated. To test this assumption, cell-free extracts of *C. albicans* and *Saccharomyces carlsbergensis* were prepared (see Methods), and the activity of the partially purified pyruvic carboxylase was measured in the presence and absence of heptamycin. The results are summarized in Table 2, where it can be seen that the activity of pyruvic carboxylase was only slightly affected by heptamycin.

Effect of heptamycin on anaerobic glycolysis of Candida albicans. An experiment was carried out with cells preincubated with heptamycin and with cells having had no such preincubation. The results, which are summarized in Fig. 4, show that anaerobic glycolysis of *C. albicans* is significantly inhibited by heptamycin. The inhibition becomes pronounced only after a lag period of 30 min.

Table 2. *Activity of pyruvic carboxylase in organisms induced and uninduced with pyruvate, in presence and absence of heptamycin*

The reaction mixture contained sodium pyruvate (NBC, Reagent) 1 M (adjusted to pH 6·0 with NaOH)—0·3 ml., citrate—NaOH buffer 0·5 M pH 6·0–0·3 ml., 100 μg. heptamycin, and 0·5 mg. of enzyme protein (total volume 3·0 ml.). The reaction mixture (without pyruvate) was incubated for 30 min. at 30° in the Warburg vessel after which time pyruvate was added (via the side arm) and CO_2 output determined for 20 min. In the absence of either the enzyme or substrate no CO_2 output was obtained even without the antibiotic.

Induction with pyruvate was carried out by suspending 24 hr. organisms (12 mg. dry weight equiv./ml.) in the chemically defined medium with 0·025 M-sodium pyruvate (instead of glucose). After 6 hr. of incubation with shaking at 30° the organisms were washed with saline and pyruvic carboxylase extracted as described (see Methods).

Enzyme source	Induction with pyruvate	Heptamycin	μl. CO_2/mg. protein in 10 min.	Inhibition (%)
Saccharomyces carlsbergensis	–	–	570	—
	–	+	530	7·1
Candida albicans no. 26	–	–	238	—
	–	+	224	5·9
C. albicans no. 26	+	–	258	—
	+	+	241	6·6

Effect of heptamycin on the oxidation of trehalose. Candida albicans strain no. 27, utilized trehalose after induction. The effect of heptamycin upon oxygen uptake with trehalose as substrate was tested on organisms preinduced and uninduced with trehalose; the results summarized in Fig. 5 show that heptamycin strongly inhibited the oxygen uptake of uninduced organisms. No inhibition of oxygen uptake by heptamycin was observed during the first 90 min. using trehalose-induced cells; upon more prolonged incubation, however, oxygen uptake was slightly decreased.

Effect of heptamycin on trehalase activity in cell-free extracts. Candida albicans, strain no. 27, grown on yeast extract + glucose agar was harvested after 24 hr. incubation, washed thoroughly with saline, and induction with trehalose carried out as described above (measured by increased oxygen uptake) (Fig. 5). Cell-free extracts were prepared with the Hughes Press from induced and uninduced organisms. Similar trehalase specific activities were noted in both preparations. In addition, the effect of heptamycin on trehalase activity was examined. Preincubation of cell-free extracts with heptamycin did not affect trehalase activity (see Table 3).

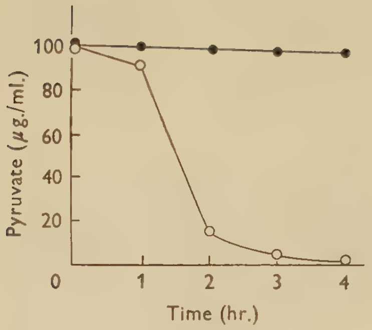

Fig. 3

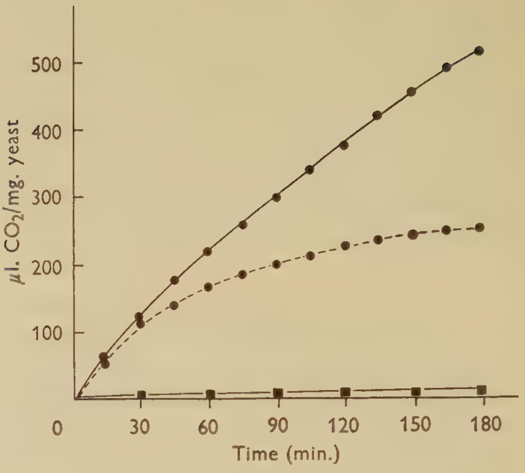

Fig. 4

Fig. 3. Effect of heptamycin on pyruvate utilization by *Candida albicans*. The reaction mixture contained 15 mg. (dry weight equiv.) *C. albicans* strain 26, 1 ml. 0·2 M-phosphate buffer pH 7·2, 300 µg. sodium pyruvate (Reagent, NBC), and 60 µg. heptamycin in a total volume of 3 ml. It was incubated 4 hr. in a 30° water bath and samples taken at intervals. The organisms were removed by centrifugation and the remaining pyruvate in the supernatant determined. ○——○, pyruvate; ●——●, pyruvate + heptamycin.

Fig. 4. Anaerobic glycolysis of *Candida albicans* in the presence and absence of heptamycin. Organisms not preincubated with the antibiotic were used, and 60 µg. heptamycin were added to the reaction mixture at zero time. The reaction mixture contained 0·5 ml. of 0·15 M-glucose, 1·0 ml. of 0·1 M-KH_2PO_4 and 12·5 mg. yeast (dry weight equiv.). Final volume—3 ml. The substrate was placed in the side arm of the Warburg vessel and was transferred to the main compartment after equilibration for 15 min. Gas phase—nitrogen. Temperature—30°. ●——●, without heptamycin; ●- - -●, with heptamycin; ■——■, endogenous glycolysis, with and without the antibiotic.

Table 3. *Trehalase activity in the presence and absence of heptamycin*

The enzyme was prepared from *Candida albicans* strain 27; the reaction mixture contained 10 mg. trehalose, 0·5 ml. phosphate buffer (pH 5·8), 40 µg. heptamycin, and 0·5 ml. (15 mg. protein) of the enzyme preparation (total volume 2·0 ml.). The reaction mixture was incubated for 4 hr. in 30° water bath. Aliquots of 1·0 ml. were taken and the protein precipitated by the addition of 1 ml. of 5 % (w/v) $ZnSO_4$ and 1·0 ml. equimolar solution of $Ba(OH)_2$. After centrifugation the reducing sugar in the supernatant was determined. The reaction mixture without enzyme and heptamycin was used as a blank.

Preinduction of cells with trehalose prior to enzyme extraction*	Trehalose in the reaction mixture	Incubation with with heptamycin	µg. glucose/mg. protein
−	+	−	320
−	−	−	10
+	+	−	325
+	−	−	11
+	+	+	316

* For details of induction experiment see Fig. 5.

Effect of heptamycin and other heptaene antibiotics on phosphate uptake by Candida albicans. Heptamycin in fungistatic concentrations (5 μg./ml.) produced immediate and complete inhibition of phosphate uptake by *C. albicans* (see Fig. 6). The other heptaenes also brought about complete inhibition of phosphate uptake at minimum inhibiting concentrations (Fig. 7). Heptamycin at 0·15 μg./ml. which only partially inhibited growth, showed a 50 % inhibition of phosphate uptake.

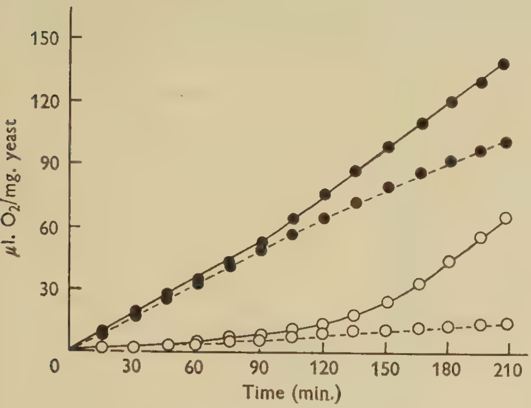

Fig. 5

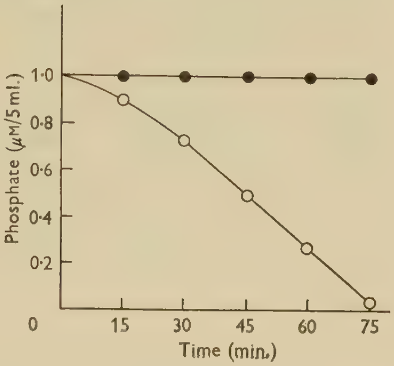

Fig. 6

Fig. 5. Effect of heptamycin on trehalose oxidation by *Candida albicans* strain 27. Induction with trehalose (0·025 M) was carried out by incubating the organisms (4 mg. dry weight equiv./ml.) in the chemically defined medium (without glucose) for 4 hr. at 30°. The organisms were then washed thoroughly and resuspended in a reaction mixture which contained 0·025 M-trehalose, and 60 μg. heptamycin in a total volume of 3 ml. The trehalose and the antibiotic were placed in the side arm and transferred to the main compartment of the Warburg vessels after an equilibration period of 15 min. *Uninduced organisms*: ○——○, trehalose; ○----○, trehalose + heptamycin. *Induced organisms*: ●——●, trehalose; ●——●, trehalose + heptamycin.

Fig. 6. Effect of heptamycin on phosphate uptake by *Candida albicans*. To the chemically defined medium containing inorganic phosphate (0·2 μM/ml.) thoroughly washed *C. albicans* organisms were added (18 hr. culture) to give a final concentration of 1·0 mg./ml. (dry weight equiv.); 100 ml. aliquots with and without heptamycin (5·0 μg./ml.) were transferred to 250 ml. Erlenmeyer flasks and incubated at 30° for 75 min. in a Ross–Kershaw shaker. Ten ml. samples were taken at intervals, cooled immediately in ice, and the organisms removed by centrifugation; 5 ml. aliquots were used to determine the remaining inorganic phosphate. ○——○, without heptamycin; ●——●, with heptamycin.

Effect of heptamycin on phosphate uptake by various yeasts. Assuming that the primary action of heptaene antibiotics was the inhibition of inorganic phosphate uptake one would expect a correlation between the degree of inhibition of phosphate uptake and the sensitivity of the various yeasts to these agents. Sensitive and resistant yeasts were examined to test this hypothesis. The same method as described above was used. The results are summarized in Table 4. A direct relation may be seen between the sensitivity of a given yeast strain to heptamycin and the minimal concentration of the antibiotic required to completely inhibit phosphate uptake. Inhibition of phosphate uptake by *Debaryomyces nicotianae*, a relatively resistant strain, was observed only at antibiotic concentrations great enough to show a fungistatic effect. A similar effect was observed with yeasts which were highly sensitive to heptamycin.

Effect of heptamycin on CO_2 output from glucose and esterification of inorganic phosphate by cell-free extracts of Candida albicans. The inhibition exhibited by heptamycin on anaerobic glycolysis and phosphate uptake in intact organisms may be due to the action of the antibiotic either within the cell or at its surface. Liberation of CO_2 from glucose and formation of organic phosphates were examined using cell-free extracts of *C. albicans* in the presence and absence of heptamycin. Figure 8 shows that heptamycin did not affect the formation of CO_2 by cell-free extracts to any significant degree. The incorporation of inorganic phosphate into sugar phosphates by cell-free extracts was also tested. Samples of the reaction mixture were examined for inorganic phosphate at the beginning and at the end of the experiment, and tests for the detection of sugar phosphates were carried out simultaneously by paper chromatography. Most of the sugar phosphate appeared as hexose diphosphate. No difference was observed in the amounts of esterified phosphates found in the presence or absence of heptamycin (Table 5).

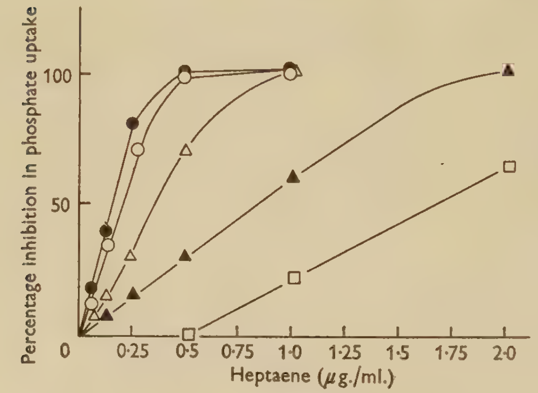

Fig. 7

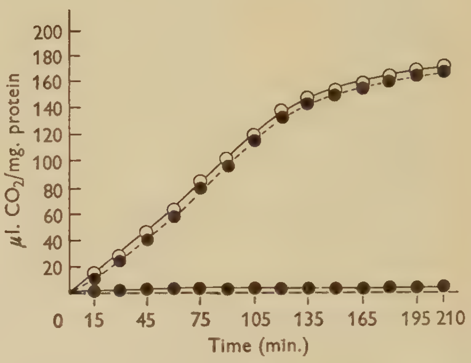

Fig. 8

Fig. 7. Inhibition of phosphate uptake by various heptaenes. Test tubes (16 × 190 mm.), each containing 5 ml. of the chemically defined medium, 0·2 μM/ml. inorganic phosphate, 3 mg. dry weight equiv. *Candida albicans* and the antibiotic indicated were incubated for 90 min. at 30° with continuous shaking. The reaction was stopped by immediately cooling the tubes in ice. The cells were removed by centrifugation and the inorganic phosphate in the supernatant fluid was estimated. Simultaneously the minimum inhibitory concentration of the various heptaenes for *C. albicans* was determined. ●——●, Heptamycin 0·5 μg./ml. MIC, see Methods for potency of heptaenes. ○——○, trichomycin 0·5 μg./ml. MIC; △——△, candicidin B 1·0 μg./ml. MIC; ▲——▲, amphotericin B 2·0 μg./ml. MIC; □——□, ascosin 6·0 μg./ml. MIC.

Fig. 8. Effect of heptamycin on CO_2 output from glucose by *Candida albicans* cell-free extract. The reaction mixture contained glucose 150 μM, phosphate buffer 150 μM (pH 5·8), heptamycin 60 μg, and cell-free extract (20 mg. protein). Total volume 3·0 ml. The glucose and the antibiotic were put in the side arm of the Warburg vessel and introduced to the main compartment after an equilibrium period of 15 min. Gas phase: air; temperature 30°. ○——○, without heptamycin; ●----●, with heptamycin; ●——●, glucose absent, with and without the antibiotic.

DISCUSSION

Heptamycin was found to greatly inhibit the oxygen uptake by *Candida albicans* when pyruvate or lactate was used as substrate, whereas only a slight effect was noted when the substrate was glucose, acetate, acetaldehyde or ethanol. It would seem that the effect of the antibiotic was due to the blocking of the active transport of pyruvate and lactate into the yeast cell, rather than to interference with metabolism inside the cell. This hypothesis is supported by the fact that the cell-free pyruvic carboxylase was not affected by heptamycin. The same explanation holds for the inhibition by heptamycin of oxygen uptake with trehalose as substrate, as trehalase activity was not affected by the antibiotic. Thus, the effect of heptamycin can be traced to its action at the cell surface. However, our experiments do not exclude the possibility that heptamycin may also inhibit the intracellular oxidative metabolism of pyruvate.

Cell-free extracts of yeast uninduced with pyruvate or trehalose contained pyruvic carboxylase and trehalase activities in amounts equal to those found in induced organisms. However, the oxidation of these substrates by intact uninduced organisms was preceded by a lag period, indicating that transport of these substrates into the cell is inducible. The effect of heptamycin may be caused by inhibition of the synthesis of the inducible permeases of pyruvate and trehalose (Cohen & Monod, 1957). On the other hand, heptamycin prevented the uptake of pyruvate but not trehalose by either uninduced or induced organisms. This difference can be explained by assuming that heptamycin inactivated the pyruvate permease of *Candida albicans*, but not the trehalose permease.

Table 4. *Comparison of the concentration of heptamycin required to inhibit growth and block phosphate uptake. The experiment was carried out as described in Fig. 7*

	Minimum concentration of heptamycin inhibiting	
Strain	Growth (μg./ml.)	Phosphate uptake (μg./ml.)
Candida albicans 26	0·5	0·5
Saccharomyces cerevisiae	0·06	0·25
Cryptococcus albidus	0·25	0·25
Debaryomyces nicotianae	65	65

Table 5. *Effect of heptamycin on esterification of inorganic phosphate by cell-free extracts of* Candida albicans

		μM inorganic phosphate (in the reaction mixture)		Phosphate esterified
Substrate	Heptamycin	0 min.	210 min.	(μM)
None	–	150	143	—
Glucose	–	150	70	73
None	+	150	143	—
Glucose	+	150	71	72

For experimental conditions see Fig. 8.

Fungistatic concentrations of heptamycin and other heptaenes caused an immediate and complete inhibition of phosphate uptake by various yeasts. Similar observations were made by Bradley (1958*b*) and by Osteux, Tran-Van-Ky & Biguet (1958). On the other hand, even high concentrations of heptamycin had no effect on esterification of inorganic phosphate in cell-free extracts of *Candida albicans*. It may thus be assumed that inhibition of phosphate uptake in intact organisms is due to interference by the heptaenes with the mechanism of phosphate transport into the cell. Goodman & Rothstein (1957) and Mitchell (1957), have shown that yeast and bacteria are equipped with mechanisms for the active uptake of inorganic phosphate presumably located at the cell membrane. The lipophilic properties of the heptaenes may explain their possible affinity for the lipoproteins of the cell membrane (Mitchell, 1957).

The effect of heptamycin may not be limited to the external cell membrane but may affect intracellular structures (e.g. mitochondria) as well. If this were so, phosphate would not only be prevented from entering the cell but also its flow through the cell compartments would be distorted (Lynen, Hartmann, Netter & Schuegraf, 1959). It is possible that the remarkable differences in effect of heptamycin on aerobic and anaerobic metabolism of glucose (Figs. 1, 4) are due to such a disturbance. Alternatively, the uptake of glucose by the cell may be different under aerobic and anaerobic conditions. However, according to Lynen *et al.* (1959) oxygen did not influence the permeability of glucose into baker's yeast.

The results described above differ from those obtained with the tetraene antibiotic, nystatin (Lampen *et al.* 1957; Bradley, 1958*a*, *b*). Thus, Lampen *et al.* (1957) found that nystatin (and amphotericin A, B) inhibited both aerobic and anaerobic utilization of glucose, the latter process (glycolysis) was somewhat more sensitive. Bradley (1958*a*) found nystatin to inhibit glucose utilization not only in intact *Candida stellatoidea* but in acetone treated organisms and cell-free extracts as well. We, on the other hand, found the oxidation of glucose to be only slightly affected by heptamycin. The possibility that nystatin binds inorganic phosphate (Bradley, 1958*b*) does not hold for heptamycin, as the uptake of inorganic phosphate in the present experiments was almost completely blocked even when the ratio of phosphate to heptamycin (w/w) was increased to 200:1 in the reaction mixture. Furthermore, as inorganic phosphate is essential to organisms both sensitive and resistant to heptamycin, no selective action of heptamycin on various yeasts was to be expected. Therefore, the mode of action of the heptaene antibiotics tested seems to differ in some respects from that of nystatin.

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A Specific Polysaccharide from *Mycoplasma mycoides*

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SUMMARY

Washed suspensions of *Mycoplasma mycoides* var. *mycoides* were found to contain *c.* 10 % by weight of carbohydrate. The only sugars detected in hydrolysates were galactose and ribose. A galactan was obtained by extraction with warm aqueous phenol. The galactan was separated from ribonucleic acid by ultracentrifugation or by treatment with an ion-exchange resin. The product was rapidly hydrolysed by acid, releasing galactose and *c.* 4 % of $CHCl_3$-soluble lipid. It formed precipitates with specific antiserum and could be used to sensitize sheep erythrocytes to agglutination by the antiserum. It was not pyrogenic for rabbits and had little in common with the lipopolysaccharides of typical Gram-negative bacteria.

INTRODUCTION

Little is known of the carbohydrates of the pleuropneumonia-like organisms (PPLO), although Kurotchkin (1937) isolated from *Mycoplasma mycoides* a serologically active fraction which gave positive Molisch and negative biuret reactions. We report here a study of the strain ('V 5') of *M. mycoides* var. *mycoides* used in this laboratory for the preparation of a vaccine against contagious bovine pleuropneumonia. Our objectives were to identify the component sugars and to examine the organism for the presence of specific polysaccharides. Warm aqueous phenol has been widely used for the extraction of lipopolysaccharides from Gram-negative bacteria (Westphal, Lüderitz & Bister, 1952). These complex materials are derived from the cell-wall structure and carry the specificity of the somatic antigens. It was of interest to apply this method to one of the Mycoplasma organisms, which lack a cell-wall of the kind found in typical bacteria (Kandler & Zehender, 1957; Plackett, 1959).

METHODS

Preparation of washed suspensions

Mycoplasma mycoides var. *mycoides* strain 'V 5' was grown in BVF-OS medium (Turner, Campbell & Dick, 1935). This consists of a pig-stomach digest of beef liver and muscle, buffered at pH 7·4 and supplemented with 10 % (v/v) of ox serum. Five-litre Florence flasks, containing 2–3 l. medium at 37°, were inoculated with 5–8 ml./l. of a 4-day culture in the same medium. They were rotated during incubation by a device of the kind described by Mitchell (1949), on which two flasks could be rotated simultaneously. The cultures were harvested after 24–28 hr. at 37°, when the number of viable particles was 10^9–10^{10}/ml., and the optical density was still increasing. At first the contents of two flasks were pooled and worked up as a single batch. Satisfactory preparations were also obtained from cultures which had been stored at 4° for up to 3 days, so that the yield from six flasks could be harvested in one operation. Harvesting was done on a steam-driven Sharples Supercentrifuge, the bowl of which was lined with a sheet of plastic material (Rodwell & Rodwell, 1954). The paste of organisms was removed from the latter with a spatula and resuspended in about 80 ml. of 0·2 M-NaCl at 4°. A large all-glass syringe was a convenient tool for resuspending the sticky paste. The organisms were resedimented at 40,000 *g* for 15 min. They were washed once or twice in cold distilled water and were then stored at $-16°$ until required.

Total carbohydrate estimations. Two colorimetric procedures were used. The orcinol + H_2SO_4 reaction was carried out following, with minor modifications, the directions of Vasseur (1948). The tubes were heated at 80° for 20 min. and the optical density measured at 500 mμ. The anthrone reaction was carried out as recommended by Trevelyan & Harrison (1952). D-galactose, being the only sugar found apart from the nucleic-acid components, was used as the standard for both procedures. Galactose added to washed suspensions was quantitatively recovered by both methods. The galactose equivalents found by the orcinol + H_2SO_4 reaction were higher than those by the anthrone method. With orcinol ribose and galactose give approximately the same extinction/mole, but with anthrone ribose gives much less colour. Unless otherwise stated, values for total carbohydrate refer to the galactose equivalent as determined by the anthrone method; they include the contribution from the nucleic acids, which amounted to about 10 % of the total in the case of the unfractionated washed suspensions.

Pentose and deoxypentose. The orcinol + HCl reagent of Militzer (1946) was used to determine pentose compounds, and the diphenylamine reagent (Dische, 1955) for deoxypentose. The appropriate purine nucleotides or nucleic acids were used as standards. Removal of nucleic acid during purification of the polysaccharide fraction was estimated by measurement of the optical density at 260 mμ in 1 cm. silica cuvettes.

Uronic acids. The carbazole test was carried out according to Dische (1947), with glucuronolactone as a reference compound.

Reducing sugars. Neutralized hydrolysates were analysed by the colorimetric procedure of Somogyi (1952). D-galactose was the standard and the heating period was 35 min.

Esterified fatty acids. The hydroxamate reaction was carried out according to Stern & Shapiro (1953), with recrystallized trimyristin as the standard.

Phosphorus. Total P was determined by the method of Fiske & SubbaRow as modified by Chen, Toribara & Warner (1956) and by Bartlett (1959).

Nitrogen. The procedure recommended by Conway (1947) was followed, except that the NH_3 determination was made by Nesslerization.

Periodate oxidations. Periodate consumption was estimated by measurement of the optical density at 290 mμ of samples of the reaction mixtures diluted tenfold with 0·05 M-sodium acetate buffer (pH 4). Solutions of sodium metaperiodate and sodium iodate were similarly diluted for use as standards. Formaldehyde was determined by means of the acetylacetone reagent B of Nash (1953) on samples which had been treated with I_2 and $Na_2S_2O_3$ to destroy excess periodate. The reaction time was 1 hr. at 38°. Some samples were analysed with a chromotropic acid reagent after removal of excess periodate with lead dithionate according to O'Dea & Gibbons (1953). The unoxidized polysaccharide gave a large blank with this reagent. When correction for the blank was made, or when the samples were treated with sodium arsenite and the formaldehyde separated by distillation, the same values were obtained as with the acetylacetone reagent.

Paper chromatography of sugars. Samples containing large amounts of protein were hydrolysed in sealed tubes with 1 ml. $0{\cdot}25\,\text{N-H}_2\text{SO}_4/0{\cdot}5$ mg total carbohydrate. The tubes were heated at 100° for 18–20 hr. After removal of sulphate as $BaSO_4$, incompletely hydrolysed protein was precipitated with ethanol as recommended by Gottschalk & Ada (1956). The overall recovery of total carbohydrate, determined by the orcinol + H_2SO_4 or the anthrone reaction, was *c.* 80 %. Hydrolysates were chromatographed on Whatman no. 1 paper by the descending technique. The following solvent systems were used: water-saturated phenol (in the presence of NH_3 and KCN), *n*-butanol + pyridine + water (6:4:3), *n*-butanol + ethanol + water (52:31:17), *n*-butanol + glacial acetic acid + water (4:1:5), *iso*-propanol + water (9:1), and water-saturated *s*-collidine. Reagents used to detect the spots included ammoniacal $AgNO_3$ (Dedonder, 1952), aniline hydrogen phthalate (Cummins & Harris, 1956), lead tetra-acetate in benzene (Buchanan, Dekker & Long, 1950) and β-indolylacetic acid (Heyrovsky, 1956).

Reversed-phase chromatography of long-chain fatty acids. Long-chain fatty acids were chromatographed on disks of Whatman no. 1 paper impregnated with a paraffin fraction (b.p. 200°–210°) as described by Nowotny, Lüderitz & Westphal (1958). Dithio-oxamide (0·03 %, w/v) in ethanol (Ballance & Crombie, 1958) was used to detect the positions of the Cu^{++} salts. OsO_4 vapour was used to reveal unsaturated acids.

Dry weights. Samples of the washed suspensions of organism were dried in an oven at 100° overnight. Analytical data for the purified polysaccharide fraction refer to material dried to constant weight *in vacuo* at 78°–80°.

Ultracentrifugation. The Spinco model L preparative ultracentrifuge was used. The rotors were precooled to 2°–4° and the rotor chamber was refrigerated during runs. The centrifugal fields are those at the centre of the tubes (average radius), and the times do not include acceleration and deceleration periods. Analytical ultracentrifugation was done in the Spinco model E.

RESULTS

Identification of the component monosaccharides

The unfractionated suspensions were found to contain total carbohydrate equivalent to *c.* 10 % of the dry weight. For a series of four batches of organisms the values ranged from 10·3 to 12·8 mg. galactose/100 mg. dry weight, the mean value being 11·2 mg. These values include the contribution from the nucleic acids, which was equivalent to about 1 mg. galactose/100 mg. dry weight. No uronic acid was detected. Only traces of hexosamine-like material are present in suspensions of *Mycoplasma mycoides* (Plackett, 1959).

When hydrolysates were chromatographed on paper it was found that most of the sugar migrated like galactose in all the solvent systems. Only two other components were detected. The most abundant of these was identified as ribose (see below), presumably derived from ribonucleic acid (RNA). A third very faint spot of high mobility appeared on papers which had been run in the butanol-containing solvents and then developed with the $AgNO_3$ reagent; this was probably glycerol (see below). To confirm the identity of the main component sugar with galactose, a hydrolysate of washed organism was fractionated on a column of cellulose powder (Hough, Jones & Wadman, 1949). Table 1 shows the recovery of material during processing of the hydrolysate. A portion of the lyophilized residue, containing 5·3 mg. pentose and 23·9 mg. galactose (anthrone method, corrected for pentose), was transferred in a small volume of water to the top of a 25 g. column of Whatman Ashless Cellulose powder which had been exhaustively washed with the eluting solvents. Pentose was eluted with acetone + water (95:5 by vol.), and hexose with methanol. The eluates were evaporated to dryness. The residue from the hexose fraction was dissolved in 1·2 ml. 50 % (v/v) ethanol in water and mixed with 0·035 ml. 10 % (v/v) acetic acid and 0·04 ml. of freshly distilled α-methylphenylhydrazine After 2 hr. at 37° and 2 hr. at 3° the precipitate was filtered off and recrystallized four times from 30 % (v/v) ethanol in water. The product melted at 189°, as did an authentic sample of D-galactose α-methylphenylhydrazone prepared in the same way. The mixed melting point was 188°.

Table 1. *Recovery of sugars after hydrolysis of washed Mycoplasma organisms*

The washed organism was hydrolysed in 0·25 N-H_2SO_4 for 18–20 hr. at 100°.

	Total galactose equivalent mg./360 mg. dry wt.	
	Orcinol + H_2SO_4 reaction	**Anthrone reaction**
Unhydrolysed suspension	55 (100)	40·5 (100)
Hydrolysate after removal of insoluble residue	48·5 (88)	38·3 (94)
After precipitation of $BaSO_4$	47·2 (86)	37·6 (93)
Concentrated supernate from ethanol precipitation	45·8 (83)	37·5 (93)
After passage through cation-exchanger IR-120 (H^+)	44·4 (81)	36·2 (89)
After lyophilization		33·8 (84)

The residue from the pentose fraction was chromatographed on paper. The pentose migrated like ribose in four different solvent systems, being clearly distinguished from arabinose and xylose. Direct comparison with lyxose was not made. A fast component was also present. A portion of the material was applied as a band to a sheet of Whatman no. 1 paper and the two components separated by ascending chromatography in n-butanol + ethanol + water. The bands were located by means of guide strips and eluted with water. The fast component migrated in the same position as glycerol in the three butanol-containing solvents. Deoxyribose ran very close to glycerol in these solvents, but was slightly faster than the unknown in n-butanol + pyridine + water and detectably slower in butanol + ethanol + water. The unknown gave a negative reaction with the diphenylamine reagent. It is unlikely that deoxyribose would survive the hydrolysis procedure. The unknown resembled glycerol in that the $AgNO_3$ spots developed only when the papers were heated, and were grey-black. Reducing sugars give brown spots when the papers are exposed to NH_3 vapour at room temperature, and these remain brownish-black for some time after heating.

Isolation of the polysaccharide fraction

Suspensions of washed organism in distilled water were subjected to three cycles of freezing and thawing, a mixture of solid CO_2 and ethanol being used as the freezing bath. The frozen and thawed suspensions, containing 5–15 mg. dry weight/ml., were buffered to pH 8 by the addition of 2-amino-2-hydroxymethylpropane-1:3 diol HCl (tris) to 0·04 M and centrifuged at 26,000 *g* for 20 min. in the cold. The supernatant fluid, which contained 50–80 % of the RNA, and less than 5 % of the galactose, was discarded, and the pellet (the 'cell-residue') was allowed to drain. Repeated washing of the cell-residue did not remove all the RNA, and little or none of the deoxyribonucleic acid (DNA) was removed under these conditions.

Extraction with aqueous phenol was carried out according to Westphal, Lüderitz & Bister (1952). A suspension of the cell-residue in distilled water, containing 2–5 mg total carbohydrate/ml., was heated with an equal volume of a phenol + water mixture (1:1) at 65°–70° for 45 min. The mixture was stirred vigorously during this period. After cooling in ice-water the phases were separated by centrifugation and the lower (phenol) layer was re-extracted once with a small volume of water at 65°–70°. The pooled aqueous layers were dialysed against distilled water to remove phenol.

Table 2. *Ultracentrifugation of dialysed aqueous phase from phenol extraction of* Mycoplasma mycoides *cell-residue*

	Total carbohydrate		
	Galactose (mg.)	Recovery (%)	Galactose ($e_{260/mg.}$)
Before centrifugation	13·2	100	5·6
After 2 centrifugations	6·4	49	1·4
After 3 centrifugations	5·7	43	0·23
After 4 centrifugations	4·1	31	0·08

Recovery of total carbohydrate for the phenol extraction step was 65–85 %. The dialysed aqueous layers contained RNA. Attempts to remove this by treatment with charcoal, RNase or Mn^{++} salts were unsuccessful. Similar difficulties were encountered by Westphal, Lüderitz, Eichenberger & Keiderling (1952), who found that the lipopolysaccharide of *Escherichia coli* could be separated from contaminating nucleic acid by differential ultracentrifugation. The polysaccharide in our preparations also sedimented more rapidly than the nucleic acid. Solutions, containing 0·5–1·5 mg. total carbohydrate/ml. in 0·1 M-sodium acetate (pH 8), were centrifuged at 105,000 *g* for 4 hr. The tube contents were separated carefully by using a teat-pipette. In the most successful runs, 80–90 % of the total carbohydrate was recovered in the fraction at the bottom of the tubes, and a sixfold purification, relative to nucleic acid, was achieved. Recovery and purification were often much lower than this, and repeated centrifugations were needed. Table 2 shows the course of a typical purification.

Preparations made in this way yielded *c.* 85% of the dry weight as reducing sugar after hydrolysis in 0·5 N-H_2SO_4 for 2 hr. at 100°. The P content was less than 0·1%, but small and variable amounts of N (0·3–0·8%) suggested contamination with non-carbohydrate material. Because of this, and since the overall yields were low, a more efficient procedure was sought. It was found that the anion-exchanger Dowex 1 (2% cross-linked, analytical grade resin), in the chloride form, retained the RNA very much more strongly than the polysaccharide. The method, which gave good yields of a product of very low nucleic acid content, was applied to several larger-scale preparations. The dialysed aqueous layers from the phenol extraction step were passed through columns of the resin (200–400 mesh) until the desired purity was obtained. The amount of resin required depended on the nucleic acid content of the solution, and was determined empirically in small-scale tests. Details of two preparations are shown in Table 3. There was some retention of the polysaccharide on the resin and excess of the latter reduced the yield correspondingly. The final column effluents were concentrated on a rotary evaporator, centrifuged to remove dust particles and dialysed exhaustively against distilled water in the cold.

Table 3. *Purification of* Mycoplasma mycoides *galactan by ion-exchange*

	Total carbohydrate		
	Galactose (mg.)	Recovery (%)	Galactose ($e_{260/\text{mg.}}$)
	Preparation L1		
Dialysed extract	125	100	9·0
Effluent from column 1 (20 g. moist resin)	107	86	4·4
Effluent from column 2 (40 g. moist resin)	99	79	0·04
	Preparation L4		
Dialysed extract	131	100	3·0
Effluent from column 1 (10 g. moist resin)	121	92	1·2
Effluent from column 2 (7 g. moist resin)	114	87	0·07

Properties of the polysaccharide fraction

Because light-scattering by the somewhat opalescent solutions contributed to the observed optical densities at 260 mμ, the amount of nucleic acid remaining in the final fractions was not determined precisely; it was certainly less than 0·5%. Preparations purified by ultracentrifugation contained rather more N, and yielded slightly less reducing sugar on hydrolysis (Table 4), than did those which had been passed through the ion-exchange columns. No other differences were observed. All preparations showed comparable activity in the haemagglutination system (Cottew, 1960).

It is possible that some fractionation of the polysaccharide occurred during purification by both methods. Examination of preparation L5 (Table 4) in the analytical ultracentrifuge revealed the presence of a small amount of slowly sedimenting material (*c.* 2S) in addition to the principal boundary. The latter was hypersharp at 0·96 % (w/v) concentration, whether centrifugation was carried out in water or in 0·2M-NaCl. Dilution to 0·24 % (w/v) increased the sedimentation constant of the main boundary, which remained markedly asymmetrical, to 19S or about twice the value found at the higher concentration. No conclusions can be drawn concerning the homogeneity of the material composing the main peak. It was shown that the serologically active material in preparation L3 moved as a single band on paper electrophoresis over the range pH 4–11 (Buttery, 1959). No further tests of homogeneity were performed.

Table 4. *Composition of* Mycoplasma mycoides *galactan*

Preparation		N (%)	P (%)	Reducing sugar* (%)	Ash (%)
S2	U†	0·3	< 0·1	86 (0·5N-H_2SO_4, 2 hr.)	—
S3	U	0·5	< 0·1	83 (0·5N-H_2SO_4, 2 hr.)	*c.* 3
X1	U	0·4	< 0·1	— —	—
L1	I†	0·15	—	89 (0·5N-H_2SO_4, 2 hr.)	2
L3	I	—	—	88 (0·1N-HCl, 1·5 hr.)	—
L4	I	< 0·1	< 0·1	91 (0·1N-HCl, 1 hr.)	—
L5	I	0·16	0·06	89 (0·1N-H_2SO_4, 2 hr.)	< 1

* Determined by the Somogyi (1952) colorimetric method with D-galactose as the standard, calculated as anhydrohexose. Hydrolysis conditions shown in parentheses.

† U = purified by ultracentrifugation, I = purified by ion-exchange.

Elementary analysis of preparation L1 was carried out by the Australian Microanalytical Service, C.S.I.R.O. Found: C 44·09, H 6·31, ash 1·99. $C_6H_{10}O_5$ requires C 44·44, H 6·22. In no case did the yield of reducing sugar on hydrolysis approach the theoretical value for a simple polysaccharide. No sugar other than galactose was detected on paper chromatograms. No heptose was detected by the cysteine-H_2SO_4 reaction (Dische, 1953); the spectrum of the reaction product closely resembled that given by an equivalent amount of galactose. 3,6-Anhydrogalactose, if present, would yield 5-hydroxy-2-furaldehyde when heated in acid. No trace of this compound, which has an absorption spectrum with peaks at 228 and 285 mμ (O'Neill, 1955), was detected in neutralized hydrolysates.

Acid-lability. The polysaccharide is very readily hydrolysed by acid. Table 5 shows the times required to obtain half the maximum yield of reducing sugar under various conditions. Data for inulin and lactose are included for comparison.

Periodate oxidation. Table 6 shows the course of periodate consumption by the polysaccharide. Samples of the reaction mixtures were treated with ethyleneglycol to remove unreacted periodate, reduced with sodium borohydride and dialysed against distilled water. The products were hydrolysed in 0·5N-HCl for 2 hr. at 100°. After removal of HCl by repeated evaporation, the hydrolysates were chromatographed in *n*-butanol + pyridine + water and in *iso*propanol + water. Authentic samples of D-galactose, L-threitol, L-arabinose, dulcitol, glycerol and ethylene glycol were run as markers. Spots corresponding in position to galactose, threitol and glycerol appeared in all the experimental samples when the papers were developed with the silver nitrate reagent. Material which had been oxidized for 1 hr. gave galactose as the major spot. After 60 hr. or longer, threitol was the most

Table 5. *Half-times for acid hydrolysis of* Mycoplasma mycoides *galactan*

	Acid	Temperature	$t_{\frac{1}{2}}$ (min.)
Preparation L 1	$0{\cdot}5$-N-H_2SO_4	74	42
		100	3·5
	$0{\cdot}01$ N-H_2SO_4	100	128
	$0{\cdot}01$ N-HCl	100	120
Inulin	$0{\cdot}01$ N-HCl	100	*c.* 1
Lactose	$0{\cdot}01$ N-HCl	100	*c.* 3×10^3

$t_{\frac{1}{2}}$ = time to attain half the maximal yield of reducing sugar.

abundant product. A rough visual comparison with mixtures of known composition indicated the presence of more than ten times as much threitol as of galactose or glycerol. In view of a recent report that glycerol may be lost during evaporation of solutions containing HCl (Hanahan & Olley, 1958), it is possible that a larger proportion of glycerol was formed.

Table 6. *Periodate oxidation of* Mycoplasma mycoides *polysaccharide*

The reaction mixture contained 2·5 mg polysaccharide preparation L4/ml. and sodium metaperiodate at an initial concentration of 0·03 M. Temperature: 5°, pH *c.* 5.

Experiment no.	Time	Mole $NaIO_4$ consumed/mole galactose
1	13 min.	0·12
	42 hr.	1·03
	73 hr.	1·05
	140 hr.	1·07
2	35 min.	0·13
	18 hr.	0·92
	46 hr.	1·04
	120 hr.	1·08

The results are consistent with a structure composed mainly of galactopyranose units linked through C4 or of galactofuranose units linked through C5 or C6. The acid-lability of the material was consistent with the furanose structure, which has been assigned to galactocarolose, an acid-labile galactan formed by *Penicillium charlesii* G. Smith (Haworth, Raistrick & Stacey, 1937). The terminal non-reducing end-groups of galactocarolose undergo rapid oxidation by periodate, with the release of formaldehyde from the primary alcohol group (O'Dea, 1953). The production of formaldehyde during oxidation of the *Mycoplasma mycoides* galactan was therefore studied. The reaction mixture contained 1·07 mg. preparation L1/ml., 0·01 M-sodium metaperiodate and 0·05 M-sodium acetate buffer (pH 3). A slow release of formaldehyde was observed, amounting to less than 1 mole/100 mole galactose during 24 hr. This was probably not due to oxidation of primary alcohol groups. It was concluded that the galactan contains few, if any, unsubstituted galactofuranosyl end-groups.

Infra-red spectrum. The infra-red spectrum of the *Mycoplasma mycoides* galactan was very poorly defined, but broad bands were present near 870 and 805 cm^{-1}. A sample of methyl β-galactofuranoside showed a rather broad band near 870 cm^{-1}, possibly arising from vibrations of types B and C (Barker & Stephens, 1954) and another broad band near 805 cm^{-1}, probably arising from their type D vibration.

In the spectrum of methyl β-galactopyranoside, bands were observed near 887, 868 and 782 cm^{-1} as reported by Barker, Bourne, Stacey & Whiffen (1954), but there was no band in the vicinity of 805 cm^{-1}. The presence of a band at this latter frequency in the spectrum of the galactan is therefore not inconsistent with a furanose structure.

Evidence for a lipid component. It was noticed that the solutions became turbid during the early stages of acid hydrolysis and tended to clear on prolonged heating. This behaviour was consistent with the presence of a lipid component. A sample (10 mg.) of preparation L4 was hydrolysed for 8 hr. at 100° with 5 ml. 5 N-HCl. The cooled solution was extracted three times with 10 ml. portions of ether. The ethereal extracts were dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in benzene for reversed-phase paper chromatography. No unsaturated acids were detected, but the copper reagent revealed two distinct bands corresponding in position to palmitic and stearic acid markers. The band which migrated like palmitic was the more intense of the two. The aqueous phase, after extraction of the fatty acids, was examined for the presence of ninhydrin-reactive compounds. No spots were detected on paper chromatograms; 0·2 % of an α-amino acid would have been readily detected.

Isolation of a lipid fraction. A sample (19·2 mg.) of preparation L4 was heated at 100° with 1·5 ml. 0·1 N-HCl under nitrogen. The solution became markedly turbid within 10 min. After 1 hr. the mixture was cooled in ice and centrifuged at 30,000 *g* for 20 min. A pale yellow pellicle formed at the surface and adhered to the walls of the tube. It was recovered as completely as possible on a small filter paper and washed with a small volume of 0·1 N-HCl and then with distilled water. The paper was dried *in vacuo* at room temperature, and the lipid extracted therefrom with ether and chloroform. After evaporation of the solvents the residue was dried *in vacuo* over P_2O_5 at room temperature; 0·5 mg. was recovered (*c.* 2·5 % of the weight of the intact polysaccharide). No sterols were detected in the lipid fraction by the Liebermann-Burchard reaction (Stadtman, 1957), but the presence of esterified fatty acids was indicated by the reaction with hydroxylamine, 100 μg. of the material giving the same optical density at 520 mμ as did 59 μg. of trimyristin. A sample of the lipid (0·25 mg.) was saponified by heating for 1 hr. with 1 ml. ethanolic KOH (Entenman, 1957) at 100°. The solution was acidified and the fatty acids extracted with ether for chromatography as described above. Bands corresponding to palmitic and stearic acids were again observed, the former being the more intense. The aqueous phase was deionized and chromatographed on paper in three different solvent systems, with glycerol and galactose as markers, the spots being revealed with the $AgNO_3$ reagent. The experimental material gave spots which moved the same distance as glycerol in all cases; no hexose was detected.

The lipid fraction could not be separated from the polysaccharide by simple solvent extraction. Preparation L5 was extracted under reflux with ethanol, ether and chloroform in that order. A sample (55·8 mg) of the dried material was then hydrolysed with 6·3 ml. 0·1 N-H_2SO_4 under nitrogen at 100° for 2 hr. The solution was cooled and extracted twice with 2 ml. portions of light petroleum (b.p. 55°–60°), and three times with 2 ml. portions of chloroform. The pooled extracts were washed three times with 1 ml. portions of water and evaporated to dryness. The residue, dried to constant weight *in vacuo* over P_2O_5 at room temperature, weighed 2·3 mg, representing 4·1 % of the dry weight of the intact preparation. It contained 0·3 % P, that is *c.* 20 % of the total P of the unhydrolysed material. Since some degradation probably occurred during hydrolysis, the total weight of lipid, and its P content, may be somewhat higher than these data indicate. However, it is clear that the lipid cannot contain more than 1·5 % P.

Optical rotation. The intact polysaccharide showed a large negative rotation; $[\alpha]_D = -140^\circ\ (\pm 3^\circ)\ (c = 1, H_2O)$, determined for preparation L5 after exhaustive solvent extraction. The equilibrium value after acid hydrolysis and removal of the lipid fraction, $[\alpha]_D = +80{\cdot}8^\circ\ (\pm 2{\cdot}4^\circ)\ (c = 0{\cdot}4, H_2O)$, shows that at least 97 % of the sugar was D-galactose (theory for 100 % D-galactose = +80·5°).

Biological properties. The serological reactions of the material are described in detail by Cottew (1960). Precipitin and indirect haemagglutination reactions were obtained with specific antisera. No pretreatment of the polysaccharide was required for sensitization of sheep erythrocytes. Heating for 1 hr. at 56° in 0·25 N-NaOH destroyed the capacity to sensitize erythrocytes, as well as the capacity to inhibit the agglutination of sensitized erythrocytes. This behaviour is in marked contrast to that reported for enterobacterial lipopolysaccharides. No significant temperature rise was observed following the injection of the *Mycoplasma mycoides* polysaccharide into rabbits at doses up to 1 μg./kg.

DISCUSSION

Further characterization of the lipid fraction and of the way in which it is linked to the carbohydrate moiety will probably require larger amounts of material than are at present available. In the absence of this information, and of data on the homogeneity of the product and the nature of the fraction (*c.* 5 %) still unaccounted for, no definite structure can be assigned to the galactose polymer. The large negative rotation indicates the β-galactosyl configuration. The observed value is in fact more negative than any reported for galactans of known structure, being approached only by that for galactocarolose ($[\alpha]_D = -84^\circ$; Haworth *et al.* 1937). The *Mycoplasma mycoides* galactan is rather more stable to acid hydrolysis than galactocarolose, but more labile than varianose, a small polysaccharide composed mainly of −1:4-β-galactopyranosyl units (Haworth, Raistrick & Stacey, 1935). Another galactan thought to consist mainly of −1:4-β-D-galactopyranosyl units was isolated from the seeds of *Strychnos nux-vomica* by Andrews, Hough & Jones (1954); this has $[\alpha]_D = +69^\circ$, and is much more stable to acid than is varianose. Since other features of the molecule may have a marked influence on hydrolysis rates, no firm conclusions concerning the ring form can be drawn from these data. Moreover, the acid-stability of the methyl galactoseptanosides is similar to that of the furanosides (Micheel & Suckfüll, 1933). Structures containing the seven-membered ring would also be consistent with the periodate data for the *M. mycoides* galactan.

The *Mycoplasma mycoides* galactan has little in common with the lipopolysaccharides of typical Gram-negative bacteria. The latter are characterized by a relatively high content of N, P and lipid and are composed of a number of different sugars and hexosamines. Apart from the observation that it inhibits the agglutination of formalized suspensions, we have no evidence that the galactan forms part of the surface structure of *M. mycoides*. The amounts of galactose present in the organisms would be sufficient for such a role. Experiments to determine the part played by the lipid and carbohydrate components in the serological reactions are in progress.

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The Influence of Proflavine on the Synthesis of Foot-and-Mouth Disease Virus

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SUMMARY

Infective ribonucleic acid (RNA) can be extracted with phenol from virus-infected pig kidney tissue-culture cells before the appearance of new complete virus. In the early stages of multiplication, however, infective RNA cannot be obtained from the cells if they are incubated with ribonuclease before treatment with phenol. This suggests that the infective RNA is in a 'free' enzyme-sensitive form at this time. At the end of the lag phase, as the RNA is incorporated into complete virus, there is a decrease in the proportion of infective RNA in the cells which is sensitive to the enzyme. Nine hours after infection all the material in the cells which yields infective RNA on treatment with phenol is insensitive to ribonuclease. Incorporation of low concentrations of proflavine into the incubation medium decreases considerably the yield of infective virus, although the amounts of infective RNA and specific complement-fixing antigen are not proportionately reduced. In the presence of proflavine the RNA in the cells remains sensitive to ribonuclease throughout the growth cycle indicating that the compound prevents the incorporation of RNA into complete virus. The complement-fixing antigen produced in the proflavine inhibited system is the 7 mμ non-infective component of the virus.

INTRODUCTION

It has recently been shown (Brown & Stewart, 1959*a*) that infective RNA can be obtained from pig kidney-tissue culture cells infected with foot-and-mouth (FMD) virus before new infective virus is formed. In the present paper it is shown that the infective RNA which can be detected in the cells in the lag phase is completely destroyed by ribonuclease. As the RNA becomes incorporated into virus there is a decrease in the proportion of infective RNA in the cells which is sensitive to the enzyme. A similar sequence of events occurs during the multiplication of tobacco mosaic virus in tobacco leaves (Engler & Schramm, 1959), and Sanders (1960) has shown that infective RNA is formed during the lag phase following infection of mouse ascites carcinoma cells with mouse encephalomyocarditis virus. This RNA, however, is present in a form which is insensitive to treatment with ribonuclease even before it is incorporated into complete virus.

It is generally considered that proflavine inhibits the formation of infective bacteriophages by blocking in some way the linkage of the phage DNA and protein which are still formed in the presence of the dye. The production of virus specific proteins, without the accompanying formation of fully infective virus, also occurs in the presence of proflavine with the viruses of poliomyelitis (Ledinko, 1958) and fowl plague (Franklin, 1958*b*). We show here that infective RNA and viral protein are still produced in the presence of proflavine in cultures of pig kidney cells infected with FMD virus although the yield of infective virus is considerably reduced. Some preliminary observations on the inhibition of FMD virus synthesis by proflavine have already been published (Brown & Stewart, 1959*b*).

METHODS

Virus and RNA preparations. Virus of strain 997 (Waldmann type C) passaged in pig kidney monolayers and harvested at 18 hr. was used for infecting the cell monolayers and suspensions. Infective RNA was prepared from the infected cells by the cold phenol method of Gierer & Schramm (1956) modified to cover the requirements of the acid-sensitive FMD virus (Brown & Stewart, 1959*a*). All the manipulations were carried out at 0–4° and the RNA preparations were assayed as quickly as possible after preparation. In all the experiments the maximum time required for phenol extraction and inoculation of the RNA on to pig kidney monolayers was 50 min. Each preparation of RNA was tested to ensure that it was free from residual virus by showing that the infectivity was destroyed by the addition of 1/10 volume of normal bovine serum or 10 μg. crystalline ribonuclease/ml.

Titration of virus and RNA. Virus was titrated throughout by inoculating 0·03 ml. doses of tenfold dilutions intraperitoneally into groups of four or five 7-day mice (Skinner, 1951). For the estimation of the amount of virus in cells or cell suspensions sodium dodecylsulphate was added to a final concentration of 0·1 % to disrupt the cells before dilution. This procedure had no effect on the titre of virus suspensions (Cartwright & Thorne, 1959). RNA was estimated by observing the cytopathic effects produced by 0·2 ml. aliquots in pig kidney monolayers (4 or 5 monolayers per tenfold dilution). The 50 % end-points were calculated by the method of Reed & Muench (1938) and the virus or RNA content expressed in ID 50/ml.

Titration of complement-fixing antigen. Brooksby's (1952) method was used. Comparison of the amounts of complement-fixing antigens present in suspensions in any individual experiment was made by determining the dilution of each suspension which fixed the same amount of complement. The harvested cells were ground in a mortar in the supernatant fluid and the debris then removed by centrifugation. Concentration of the fluids was achieved by dialysis against polyethylene glycol (Kohn, 1959).

Infection of pig kidney cell cultures. Primary cultures of pig kidney cells were suspended in Hanks' saline containing 0·5 % lactalbumin hydrolysate and 0·01 % yeast extract. Suspensions containing $2\text{–}4 \times 10^6$ cells/ml. were infected to give a multiplicity of infection of 1. After 30 min. at 37°, the cells were washed three times and resuspended in the same medium so that the final concentration of cells was approximately 2×10^5/ml. Samples of the suspensions were removed at intervals for estimations of virus and infective RNA.

Detection of 'free' RNA in infected cells. Samples of the infected cell suspensions were centrifuged, the supernatant removed and the cells resuspended in modified Hanks's saline. The suspensions were then divided into two portions. One was cooled to 0° and then extracted with phenol; the other was ground in a mortar after the addition of ribonuclease (20 μg./ml.), held at 20° for 15 min., then cooled to 0° and extracted with phenol. The first procedure yields total RNA, whereas the second procedure gives RNA which is resistant to ribonuclease when it is present in the cells. The difference between the two estimations was taken as 'free RNA', although it is not known whether the RNA is really free or bound to some other material.

Proflavine experiments. Because of the photodynamic action of proflavine on the virus (Galloway, 1937) subdued light was used in all the experiments involving the use of this compound.

RESULTS

Relationship between virus and RNA titres

The accuracy of the titration methods and the reproducibility of the phenol method for extracting infective RNA from the cells were determined experimentally. Four aliquots of virus-infected pig kidney cells, taken after 7 hr. incubation at 37°, were cooled to 0° and extracted with phenol. Each was then titrated in pig kidney-cell monolayers. One of the RNA preparations was also titrated four times. The virus content of the cells was estimated by four separate titrations in mice after first disrupting the cells with sodium dodecylsulphate. Values of $10^{7\cdot0}$, $10^{6\cdot8}$, $10^{6\cdot8}$, and $10^{6\cdot5}$ ID50/ml. were obtained for the virus titrations and $10^{2\cdot2}$, $10^{2\cdot0}$, $10^{2\cdot0}$ and $10^{1\cdot7}$ ID50/ml. for the RNA titration. In addition, the preparation of RNA which was titrated four times gave values of $10^{2\cdot2}$, $10^{2\cdot0}$, $10^{2\cdot0}$ and $10^{1\cdot7}$ ID50/ml.

In this and other unpublished work it has been found that standard virus preparations containing $10^{7\cdot0}$ ID50/ml. yield about $10^{2\cdot0}$ ID50/ml. infective RNA on treatment with phenol. Similarly $10^{6\cdot0}$ ID50/ml. of virus give about $10^{1\cdot0}$ ID50/ml. of RNA. With virus suspensions containing $10^{5\cdot0}$ ID50/ml. however, infective RNA has generally been detected infrequently in the phenol extracts and then only in one out of four or five monolayers inoculated with the undiluted extract. In the lag phase of the growth cycle, however, when the major contribution to the infective RNA content of the phenol extract is made by viral precursors, infective RNA can be detected in extracts of cells which have much lower infectivity titres (see Fig. 1*a*).

Growth of the virus and its infective RNA

There is an initial lag phase of about 1·5 hr. (measured from the end of the adsorption period) before the infectivity titre of the suspension starts to increase. Treatment of the infected cells with phenol during the lag phase yielded infective RNA, usually about 0·25–0·5 hr. before the virus titre started to rise (Fig. 1*a*). All the infective RNA present in the cells at this stage was rendered non-infective by treatment of the ground cells with ribonuclease (Fig. 1*b*). With the appearance of complete virus in the cells, however, there was a decrease in the proportion of 'free' RNA. During the 4 hr. period when the virus titre was increasing most rapidly about 80–90 % of the RNA was in the 'free' state. As the rate of virus synthesis decreased, the percentage of 'free' RNA declined rapidly and after 9 hr. all the infective RNA in the cells was resistant to ribonuclease. Virus synthesis had finished by this time (Fig. 1*a*). The complement-fixing titre of the suspension reached its maximum at about the same time, affording further evidence for this conclusion.

Control experiments showed that the action of the added ribonuclease was stopped immediately on addition of the incubated mixture of ground cells and enzyme to the cold phenol. Consequently the lower yield of infective RNA, obtained after treatment of the cells with ribonuclease, cannot be accounted for by enzymic destruction of the RNA after release by phenol treatment.

In contrast to the results obtained with the cells, treatment of the supernatant with ribonuclease during the early stages of virus growth did not lead to a diminution in the yield of infective RNA. It appears, therefore, that the cells do not discharge 'free' RNA into the medium along with complete virus unless any 'free' RNA released by the cells is destroyed rapidly in the medium.

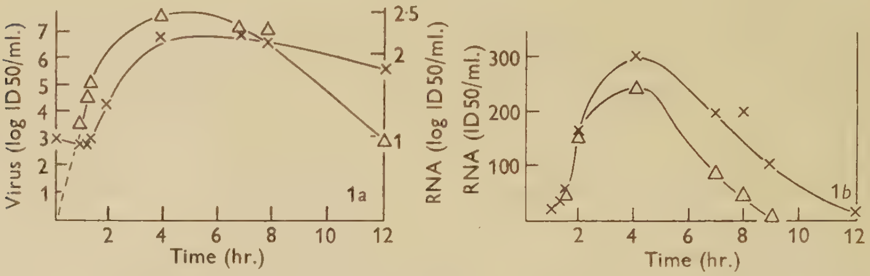

Fig. 1. (*a*) Intracellular production of infective virus (×—×) and infective RNA (△—△) in pig kidney cells infected with FMD virus. (*b*) Relative amounts of total infective RNA (×—×) and ribonuclease sensitive RNA (△—△) in the cells during the growth cycle.

Effect of proflavine on virus and RNA synthesis in pig kidney cells

The marked inhibition of virus growth which occurs on incubating FMD virus-infected cell monolayers in medium containing proflavine was described in a preliminary report (Brown & Stewart, 1959*b*). When suspensions of cells were used instead of monolayers, a similar though less-marked inhibition of virus production was observed. For example, at a concentration of 1·5 μg. proflavine/ml. the yield of virus at 4 hr. was 2·0–2·5 logs lower than in control cultures in monolayers while in suspensions the inhibition was 1·5–2·0 logs. This difference is probably related to the different concentrations of cells in the two systems as the inhibition of virus production in the presence of 1·5 μg. proflavine/ml. can be eliminated by increasing the concentration of cells to 2×10^6/ml.

Figure 2 shows typical results with virus-infected cell suspensions containing 0–2 μg. proflavine/ml. Most of the experiments have been made in this range of concentration as the yield of virus was reduced by a factor of 10 or more without a proportionate reduction in the yield of infective RNA and viral protein. In forty experiments the ratio $\log_{10}$ virus:$\log_{10}$ RNA with suspensions harvested at 4 hr. was 3 or less in suspensions containing 1·5–2·0 μg. proflavine/ml., whereas the ratio in control cultures without proflavine was 4 to 5. Figure 2 also shows that the infective RNA was produced as early in the growth cycle in the pressure of 1·5 μg. proflavine/ml. as in uninhibited cultures.

About 50 % of the infective RNA which could be obtained from control infected cells at 7 hr. was in a form resistant to the action of ribonuclease (Table 1 and

Fig. 1*b*). However, we were unable to detect any infective RNA in the phenol extracts prepared from virus-infected cells which had been incubated in the presence of 1·5 μg. proflavine/ml., if these cells were first ground with ribonuclease before extraction. The amount of ribonuclease-resistant RNA in the proflavine inhibited cells must therefore be less than 1 % of the total infective RNA. The amount of virus present in the proflavine-inhibited culture at this time ($10^{5\cdot0}$ ID 50/ml.) is just enough to yield detectable infective RNA with the techniques used in this work.

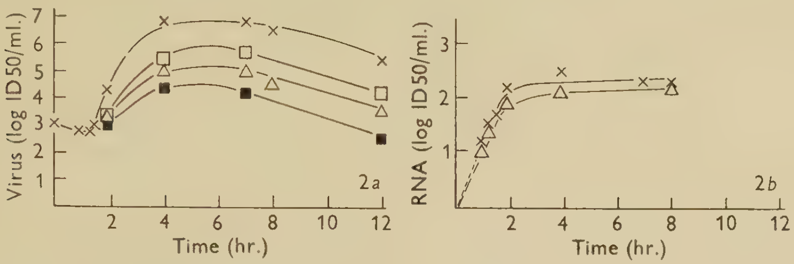

Fig. 2. (*a*) Production of virus in pig kidney cells following infection with FMD virus and incubation in the presence of proflavine: Control (×—×); 1·0 μg./ml. (□—□); 1·5 μg./ml. (△—△); 2·0 μg./ml. (■—■). (*b*) Effect of proflavine on the production of infective RNA by pig kidney cells infected with FMD virus: Control (×—×); 1·5 μg./ml. (△—△).

Table 1. *Effect of proflavine on the production of virus and infective RNA by FMD-virus infected pig kidney cells*

	Virus titre (log ID 50/ml.)		RNA titre (log ID 50/ml.)		RNAse-resistant RNA titre (log ID 50/ml.)	
Medium	4 hr.	7 hr.	4 hr.	7 hr.	4 hr.	7 hr.
Control	6·8	6·8	2·5	2·3	1·7	2·0
1·5 μg. proflavine/ml.	5·0	5·0	2·2	2·2	Nil	Nil

Effect of proflavine on protein synthesis

In the preliminary work with monolayers, virus specific complement-fixing antigens could not be detected in the 24 hr. harvests from the infected cell monolayers incubated in the presence of 2 μg. proflavine/ml., even after five-fold concentration of the material. With virus-infected suspensions incubated in the same concentration of proflavine only a trace of complement-fixing antigen was detected. In no case was there more than 5 % of the activity of the control culture. In the presence of 1·5 μg proflavine/ml., however, about 50 % of the normal yield of complement-fixing antigen was formed. It was possible to compare the yields of complement-fixing antigens only after 4 or more hours incubation. Before that time the amount present, even after concentrating the control cultures, was usually insufficient to determine with accuracy. Table 2 shows the relative amounts of complement fixed at several time intervals during the multiplication cycle. Compared with the control, the amount of antigen formed in the presence of a fixed concentration of proflavine did not alter significantly during the growth cycle.

Identity of complement-fixing antigen in proflavine inhibited cultures

Suspensions of FMD virus prepared from infected animal tissues or tissue cultures contain two complement-fixing antigens: the infective 20 mμ component and a 7 mμ non-infective component. The effect of proflavine on the synthesis of these two components was studied in two ways:

Table 2. *Effect of proflavine on the complement-fixing activity of virus infected cell suspensions at different stages of multiplication*

Proflavine (μg. /ml.)	ml. 1/30 complement fixed by 1·0 ml. antigen			
	4 hr.	6 hr.	7·5 hr.	24 hr.
0	0·07	0·16	0·20	0·28
1·0	—	—	0·10	0·14
1·5	0·04	0·08	0·09	0·12
2·0	—	—	0·01	0·01

(*a*) *Agar gel diffusion.* This is a simple qualitative method for determining the antigens present in FMD virus suspensions (Brown & Crick, 1958). It was first necessary to concentrate the harvested tissue culture fluids about 100-fold by dialysis against polyethylene glycol (Kohn, 1959). The volumes of the concentrates were adjusted so that they had the same complement-fixing titre, and then diffused from adjacent cups towards a cup containing the homotypic hyperimmune guinea-pig serum. The control virus suspension (A and D) produced two precipitin bands, whereas the suspension containing 1·5 μg, proflavine/ml. (B) produced one band identical with that formed by the 7 mμ component of the control (C and E), together with a very faint 20 mμ component band (Fig. 3). To determine whether proflavine had any effect on the precipitin band pattern produced by normal virus suspensions, 2 μg./ml. of the dye was added to the control virus suspension and allowed to stand on the laboratory bench for 3 hr. The virus suspension treated in this way (F) gave a precipitin pattern identical with that of the untreated concentrate although it was then non-infective for mice.

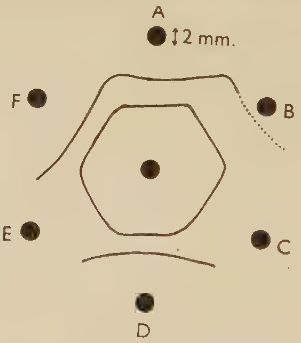

Fig. 3. Diagrammatic representation of the agar precipitin pattern obtained with virus antigens produced by pig kidney cells in the presence of proflavine: A and D—standard virus preparation; B—virus antigens obtained in the presence of 1·5 μg. proflavine/ml.; C and E—7 mμ component separated from virus; F—standard virus preparation incubated with 2 μg. proflavine/ml.

(*b*) *Effect on neutralizing activity of antiserum.* The fluids harvested from cell suspensions incubated for 24 hr. in normal medium or medium containing 1·5 μg. proflavine/ml. were rendered non-infective by treatment with acetylethyleneimine (Brown & Crick, 1959). This compound inactivates FMD virus without altering its serological structure as determined by agar diffusion. The inactivated suspensions were dialysed free from the imine and incubated with 1/1000 hyperimmune guinea-pig serum at 37° for 3 hr. Aliquots of the mixtures were then incubated with dilutions of virus at 37° for 1 hr. and titrated in mice. The reduction of the neutralizing activity of the serum by the inactivated suspensions was in direct relation to the initial virus content of the suspensions (Table 3). Whereas 1/1000 serum lowered the titre of the test virus from $10^{6\cdot8}$ to $10^{3\cdot3}$ ID50/ml. (i.e. a neutralizing titre of $10^{3\cdot5}$) a mixture of the same dilution of serum and the inactivated control virus suspension did not lower the titre of the test virus, showing that all the neutralizing activity of the serum had been blocked by the inactivated virus suspension. In contrast the proflavine inhibited suspension, although containing half as much complement fixing antigen as the control suspension, lowered the neutralizing titre of the serum by only $10^{1\cdot7}$. This result indicates the absence from the proflavine inhibited suspensions of a non-infective antigen capable of reducing the virus neutralizing antibody titre of homotypic antiserum. The 7 mμ component of the virus separated from the control virus suspension by ultracentrifugation and subsequently treated with acetylethyleneimine to remove traces of residual infective virus, had only a slight effect on the neutralizing antibody titre of the serum (Table 3).

Influence of proflavine on the cytopathic effects produced in pig kidney cells by foot-and-mouth disease virus

When pig kidney monolayers were infected with virus and treated immediately with proflavine, cytopathic changes occurred similar to those observed in untreated, infected cells. In order to examine these changes in the two systems, pig kidney cells were grown as monolayers on coverslips, infected with virus and incubated in growth medium with or without proflavine. Coverslips were removed at intervals after infection and the cells fixed and stained with Giemsa or haematoxylin. During the lag period no changes in the stained appearance of the cells could be detected in either system, but after 2 hr. cells in the absence of proflavine showed marked cytopathic changes with increased nuclear pyknosis and shrinkage of the cytoplasm. Similar changes occurred in the presence of proflavine, but these were detected only after about 4 hr. incubation. In both cases, however, 24 hr. incubation resulted in almost complete degeneration of all the cells, accompanied by an increase in virus titre only in cells free from proflavine. (Cytopathic changes in infected cells incubated with proflavine occurred without similar increase in virus titre.)

This lack of correlation between cellular injury and virus increase has also been seen with HeLa cells infected with poliovirus and subsequently incubated in the presence of proflavine (Ledinko, 1958) or *p*-fluorophenylalanine (Ackermann, Rabson & Kurtz, 1954).

Effect of proflavine on infectivity of virus suspensions in the presence of light

As Galloway (1937) has shown that proflavine has a photodynamic action on suspensions of FMD virus, it was necessary to use subdued lighting conditions. This aspect of the action of proflavine on viruses appears to have been overlooked recently despite the evidence put forward nearly 30 years ago that the dye had a photodynamic action on several phages and viruses (Burnet, 1933). The recent work of Kaufman & Hiatt (1959) with T_2 coliphage emphasizes this point.

The subdued lighting used in the present work was controlled as follows. Virus suspensions were held at room temperature for 2 hr. with or without the addition of 2 μg. proflavine/ml., in daylight or in subdued light. The suspensions were then compared for virus infectivity and also for the yield of infective RNA on treatment with phenol. The destruction of virus infectivity in the presence of proflavine was accompanied by the loss of infective RNA (Table 4). This also shows that the reduction of virus yield in infected cells in the presence of proflavine without the proportionate reduction in RNA titre is not due to a differential photodynamic effect. In the lighting conditions used in the experiments, no loss of virus or RNA infectivity was encountered.

Table 3. *Effect of addition of viral antigens on the virus neutralizing titre of hyperimmune guinea-pig antiserum*

Antigen	Virus titre of antigen* (log ID 50/ml.)	Virus titre of mixture† (log ID 50/ml.)	Diminution of neutralizing activity of serum (log ID 50)
Control virus	7·0	6·8	3·5
Control at 1/10	6·0	6·0	2·7
Antigen produced in presence of 1·5 μg proflavine/ml.	5·2	5·0	1·7
7 mμ component	2·0	3·5	0·2
Buffer control	—	3·3	—

Test virus diluted with an equal vol. phosphate buffer contained $10^{6\cdot8}$ ID 50/ml. This titre was reduced to $10^{3\cdot3}$ ID 50/ml. by 1/1000 serum, i.e. neutralization titre of serum = $10^{3\cdot5}$.

* All antigens were inactivated with acetylethyleneimine before use in the test. The titre shown was the maximum achieved during the growth cycle.

† Mixture = (inactivated antigen + 1/1000 antiserum) + virus dilutions.

Table 4. *Effect of proflavine on the titre of virus suspensions*

Treatment	Virus titre (log ID 50/ml.)	Titre of RNA (log ID 50/ml.)
None	5·8	1·2
2 μg. proflavine in daylight	Non-infective	None-infective
2 μg. proflavine in subdued light	5·8	1·3

DISCUSSION

It is generally considered, by analogy with the results obtained with bacteriophage, that animal and plant viruses break down into smaller units as they penetrate the susceptible host cell. Apart from the results of Hoyle and his colleagues (e.g. Hoyle & Frisch-Niggemeyer, 1955; Hoyle & Finter, 1957) with influenza virus and the recent findings of Sanders (1960) with mouse encephalomyocarditis virus, however, there is little direct experimental evidence to support this view, although the recent findings that RNA, isolated from a number of different animal viruses, can initiate in a suitable host an infection indistinguishable from that produced by the parent virus has rendered the hypothesis more attractive. We provide further evidence in favour of the hypothesis in the case of FMD virus and also confirm that the employment of biochemical inhibitors may be useful in elucidating the mechanism of virus synthesis. The demonstration of infective RNA in pig kidney cells infected with foot-and-mouth disease virus before the appearance of new virus suggested that the RNA and viral protein were formed separately in the cell before they combined to yield complete virus. This suggestion was strengthened in the case of the RNA by the observation that ribonuclease treatment of the cells at this stage of virus synthesis destroyed the RNA, indicating that it was in a free, enzyme-sensitive form. As the RNA became incorporated into virus this sensitivity to ribonuclease decreased. The virus itself was not affected by the enzyme. This is similar to the finding of Engler & Schramm (1959) with tobacco mosaic virus. The results of Sanders (1960) with mouse encephalomyocarditis virus suggest that the multiplication of this virus is different from that of tobacco mosaic or FMD, as the infective RNA in this case is in a form which is insusceptible to ribonuclease even before it is incorporated into complete virus.

Proflavine inhibits the growth of bacterial viruses by blocking some process or processes in the latter half of the lag phase concerned with the synthesis of the fully infective virus particle (De Mars, Luria, Fisher & Levinthal, 1953; de Mars, 1955; Kellenberger & Séchaud, 1957; Kay, 1959). As proflavine is regarded as inhibiting cytoplasmic protein synthesis, the possibility of a differential block on the synthesis of virus subunits was investigated, following the interesting results obtained by Franklin (1958*b*) with fowl plague virus and Ledinko (1958) with poliovirus. Franklin showed that S-antigen production in chick embryo tissue culture cells was not affected by concentrations of proflavine which inhibited the production of infectious particles and haemagglutinin. These observations fitted in well with the observations of Breitenfeld & Schäfer (1957) and Franklin (1958*a*) that the S-antigen multiplies in the nucleus and the haemagglutinin in the cytoplasm. Ledinko, on the other hand, showed that the yield of infectious poliovirus was considerably reduced by proflavine although the number of poliovirus-like particles counted by electron microscopy and the complement-fixing activity were the same as the untreated controls.

In the present work with FMD virus, it has been shown that proflavine has a differential effect on the synthesis of infective RNA, virus protein and complete virus. Whereas virus infected cell suspensions incubated in the presence of 1·5 μg. proflavine/ml. produced between 1 and 10 % of the normal yield of infective virus, the reduction in the yield of infective RNA and viral protein was only about twofold.

The viral protein which is synthesized in the presence of proflavine is the non-infective 7 mμ component of the virus and there is no evidence for the production of any other specific antigen. Furthermore, the infective RNA formed in the presence of the dye is not incorporated into a form which is resistant to the action of ribonuclease, as we have failed to obtain any infective RNA from proflavine inhibited cells if these are first treated with ribonuclease before extraction with phenol. It seems, therefore, that whereas in the multiplication of virus in an uninhibited system the 7 mμ component and the infective RNA can combine to form the fully infective 20 mμ virus particle, in the presence of proflavine the infective RNA and 7 mμ component are prevented from combining.

We wish to thank Dr I. A. Galloway for drawing our attention to the photodynamic action of proflavine on the virus and Mr B. Cartwright for carrying out the complement-fixation tests.

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An Immunological Study of *Bacillus subtilis* Penicillinases

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SUMMARY

Antisera to the penicillinases of *Bacillus subtilis* strains 749 and 6346 were prepared, and the neutralization of enzymic activity by homologous and heterologous antisera was studied. Antisera to 749 penicillinase had, as expected, greater neutralizing power against 749 penicillinase than against 6346 penicillinase. A 6346 antiserum, however, neutralized the heterologous enzyme more effectively than the homologous enzyme, and was also more effective in precipitating heterologous than homologous enzymic activity. The penicillinases of *B. subtilis*, unlike those of *B. cereus*, could not be adsorbed on glass, but could be partially purified by calcium phosphate gel-adsorption. There was no cross-reaction, as measured by enzyme neutralization, between the penicillinases of *B. subtilis* and that of *B. cereus* 569. Materials other than penicillinase in the enzyme preparations used did not interfere with the neutralization reactions between *B. subtilis* penicillinases and their antisera. The basal penicillinase produced by *B. subtilis* 749 had the same neutralization curve, with a homologous antiserum, as did the induced enzyme.

INTRODUCTION

Immunological techniques have yielded results of great interest in studies of bacterial enzyme synthesis (see review by Cinader, 1957). Pollock (1956*a*) used the technique of specific enzyme neutralization by antibody to determine the immunological relations between the penicillinases of different *Bacillus cereus* strains. In a further study (Pollock, 1956*b*), antisera to the penicillinase of *B. cereus* 569 were used to examine the location and properties of the cell-bound penicillinase produced by this organism. Recent results in this laboratory have suggested that similar immunological studies could profitably be carried out on the penicillinases produced by different strains of *B. subtilis*. In *B. cereus*, only about 10% of the total penicillinase formed is cell-bound, and the ratio of extracellular to cell-bound enzyme is constant throughout growth (Pollock, 1956*b*). In contrast, the penicillinase formed by growing *B. subtilis* in the first 3 hr. after induction is almost totally cell-bound, and thereafter is slowly released into the external medium (Pollock, 1961). This organism, then, seemed especially suited for studies of the mechanism of penicillinase release, and it was felt that an antiserum to *B. subtilis* penicillinase would be of value in determining the location of the cell-bound enzyme and the properties of this and of the extracellular enzyme. This paper describes some of the properties of penicillinases from two strains of *B. subtilis*, the preparation of antisera to these

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enzymes, and the effect of these antisera on penicillinase action. Immunological comparison has been made between the penicillinases of *B. subtilis* strains 6346 and 749 and the penicillinase from *B. cereus* 569; in addition, it has been possible to compare immunologically both the induced and the basal penicillinases formed by *B. subtilis* strain 749.

METHODS

Bacteria. Bacillus subtilis 6346 was a gift from the late Dr E. Duthie; strain 749 was obtained from Distillers Company Limited, Liverpool.

Growth media. For strain 6346 a modification of the casein hydrolysate medium described by Kogut, Pollock & Tridgell (1956) was used. Citrate was omitted, and trace metals ('oligodynamic solution' used by Pollock & Kramer (1958), 0·1 ml./100 ml. medium) were added. For strain 749, this medium was supplemented with 0·02 M-citrate. Spores of each strain were incubated in 20 ml. growth medium, statically overnight at 35° in 100 ml. Erlenmeyer flasks and then shaken on a reciprocal shaker (88 cycles/min., lateral throw of 3 in.) at 35° until a culture density of about 1 mg./ml. was reached. Ten ml. of such a culture were added to each of several 1 l. lots (in 5 l. flasks) of the same medium containing 0·5 % glucose (for strain 6346) or 0·1 % glucose (for strain 749). After 3 hr., when the culture density was 0·05–0·1 mg./ml., 0·1 unit/ml. benzyl penicillin was added; and 2 hr. later, at a culture density of 0·5–0·7 mg./ml., 0·5 units/ml. (strain 6346) or 1·0 unit/ml. (strain 749) penicillin were added. Incubation was continued overnight (11–16 hr.). The 6346 culture supernatant fluid contained about 20 units penicillinase activity/ml., measured manometrically (Henry & Housewright, 1947; Pollock, 1950), and the 749 culture supernatant fluid contained about 225 units/ml.

Concentration of Bacillus subtilis *penicillinases.* Preliminary investigations showed that adsorption by glass, which had proved most useful in purifying the penicillinase of *B. cereus* (Kogut *et al.* 1956) could not be applied to *B. subtilis* penicillinase. The addition of even a large amount of glass powder (1·0 g. to 3·0 ml. culture medium, over the pH range 5·5–8·1) caused no diminution in the amount of soluble penicillinase present. The penicillinase in strain 6346 culture supernatant fluid was concentrated roughly 10-fold by adding to the supernatant (adjusted to pH 5·5) 1/10 volume calcium phosphate gel (prepared according to Keilin & Hartree, 1938; 19 mg./ml.), shaking intermittently for 1–2 hr. at room temperature and centrifuging. This procedure removed 90 % of the penicillinase activity from the supernatant fluid. The gel was extracted three times with 1/5 its volume M-phosphate buffer (KH_2PO_4–Na_2HPO_4; pH 7·0), the extracts dialysed against distilled water at 0° until free from phosphate, and lyophilized. In a typical procedure, 1800 ml. of culture medium with activity 24·2 units/ml. yielded 244 mg. dried material with 118 units/mg. activity, a recovery of 66 %.

Calcium phosphate gel-adsorption was not a convenient method for concentrating 749 penicillinase. Approximately the same amount of penicillinase activity, per mg. gel, was adsorbed from the 749 as from the 6346 supernatants. Since the 749 cultures had ten times as much penicillinase activity, adsorption and elution, as described above, did not decrease the volume of solution containing enzymic activity. Strain 749 culture supernatant fluids were concentrated 10-fold by evaporating under reduced pressure in a Craig rotary evaporator at 30–35° (Craig, Gregory

& Hausmann, 1950). After dialysis, the soluble material was lyophilized. The 749 penicillinase was quite stable during this treatment. Five and one-half l. of culture medium containing 223 units activity/ml. yielded 1·65 g. dried material with 605 units activity/mg., an 80 % recovery.

Uninduced enzyme preparation. For certain control experiments cultures of strains 6346 and 749 were prepared as outlined above, but without addition of penicillin. The culture supernatant fluids were concentrated in the same manner as those of induced cultures.

Immunization. (*a*) 6346 enzyme. Twenty-one mg. of a 6346 enzyme preparation (118 units activity/mg.) in a volume of 1·0 ml. Freund's adjuvant (Freund & McDermott, 1942; modified by the use of lanolin and light liquid paraffin B.P. instead of 'Aquaphor' and heavy paraffin oil) were injected into each thigh muscle of two rabbits (6 A and 6 B). After 4 weeks, each rabbit was given 13 mg. enzyme preparation intravenously (8 mg. 'alum-precipitated' enzyme/ml. prepared as described by Pollock, 1956*a*) over a period of a week. At this point, neutralization titres were still very low, and each rabbit was re-injected intramuscularly with about 10 mg. enzyme preparation in adjuvant. One month later, the rabbits were each given 45 mg. enzyme preparation (57 units/mg.) intravenously, as before, over a 2-week-period and after 1 week bled out and the sera separated. The sera are referred to as antisera 6 A and 6 B.

(*b*) 749 enzyme. Either 150 or 125 mg. enzyme preparation (607 units/mg.) in adjuvant were injected into each thigh muscle of two rabbits (9 W and 9 S, respectively). After 6 weeks (9 W) or 3 weeks (9 S) each rabbit was injected intramuscularly with a further 23 mg. enzyme preparation in adjuvant. Four weeks later each rabbit was injected intravenously with about 200 mg. alum-precipitated enzyme over an additional 3-week period and then bled out. The separated antisera are referred to as antisera 9 W and 9 S. For the experiments reported here, the sera were not treated further. They showed no loss of neutralizing activity after being stored, frozen, for over 6 months.

Neutralization tests. Most neutralization tests were carried out in the Warburg vessel in which penicillinase activity was subsequently measured (Henry & Housewright, 1947; Pollock, 1950). The enzyme and antiserum were mixed, usually in a total volume of 0·6 ml., and left together at room temperature for 20 min. before adding bicarbonate, oxine gelatin (0·00083 M-oxine in 1 %, w/v, gelatin) and water to a volume of 2·5 ml. It was found later, however, that the degree of neutralization was the same when the other components were added to enzyme and antiserum immediately after mixing. The period of contact between enzyme (6346 or 749 penicillinase) and antiserum (9 W) before addition of penicillin could be decreased to 10 min., i.e. the time necessary for gassing and equilibration, or increased to 70 min. without changing the observed degree of enzyme neutralization.

RESULTS

Enzyme neutralization with 749 *antisera.* The neutralization curves obtained when two different amounts of 749 penicillinase were each treated with increasing amounts of antiserum 9 W are shown in Fig. 1. Apart from the lower neutralization titre of the antiserum (units of enzyme neutralized per ml. of antiserum under conditions of enzyme excess, calculated from the slope of the linear portion of the neutralization curve) against the homologous enzyme, a significant difference must be noted between these and the curves obtained by Pollock (1956*a*) for purified *Bacillus cereus* 569 penicillinase and its antiserum. The latter neutralization curve was rectilinear over approximately 95 % of its course, whereas with the *B. subtilis* 749

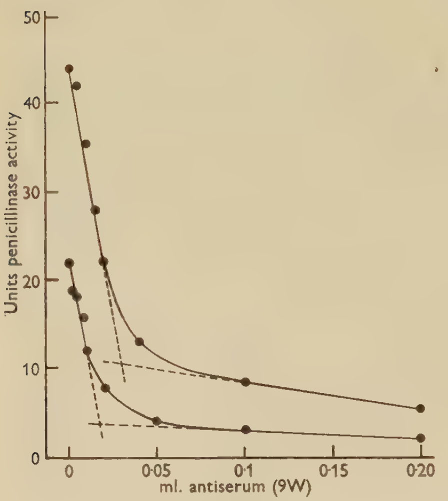

Fig. 1

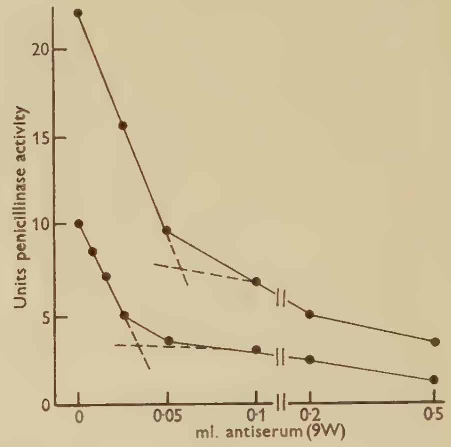

Fig. 2

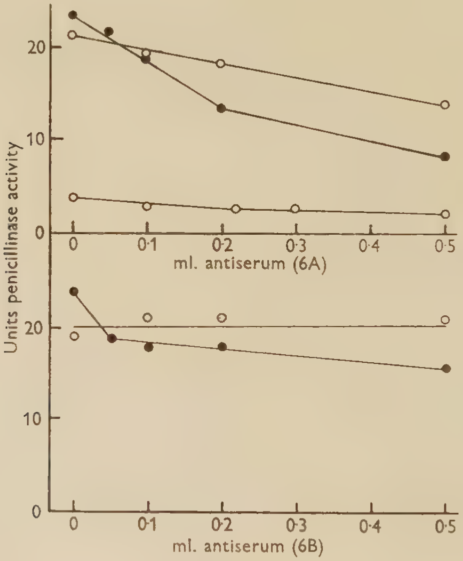

Fig. 3

Fig. 1. Neutralization curves of 749 penicillinase with 749 antiserum (9 W). Details as given in 'Methods'.

Fig. 2. Neutralization of 6346 penicillinase by 749 antiserum (9 W).

Fig. 3. Neutralization of 749 ●—● and 6346 ○—○ penicillinases with 6346 antisera (6 A and 6 B).

enzyme it became curvilinear when only about 50 % of penicillinase activity was neutralized and approached a residual value of 10 % of the original activity with excess antibody. An approximation of the equivalence point for each amount of enzyme was obtained, as shown, by extrapolating the linear portion of the curve to meet a line joining the points in the region of antibody excess. The value found by this method ($1\cdot33 \times 10^3$ units penicillinase per ml. antiserum) agreed, within 20 %, with that found by precipitation tests (Table 2). The two curves shown in Fig. 1 are parallel, indicating that the neutralization titre of the serum does not change with changing enzyme concentration.

Table 1. *Cross-reactions between the penicillinases of* Bacillus subtilis *and* Bacillus cereus *measured by enzyme neutralization*

The figures represent neutralization titre (units penicillinase neutralized by 1 ml. serum measured in the linear portion of the neutralization curve).

	Antiserum			
Enzyme	749 (9 W)	346 (6 A)	569*	Normal rabbit serum
B. subtilis 6346	244	15	0	0
749	1085	50	0	0
B. cereus 569	0	0	21,300	0

* Rabbit 362 (Pollock, 1956*a*).

When 6346 penicillinase was treated with antiserum 9 W the neutralization curves obtained were similar in form to those obtained with the homologous enzyme (Fig. 2), but the neutralization titre of the 749 antiserum toward the 749 enzyme was 4·8 times as great as that of 749 antiserum toward the 6346 enzyme. Calculation of the equivalence point, as shown in Fig. 2, gives a mean value of $0\cdot32 \times 10^3$ units/ml. The value determined for the homologous inhibition is 4·2 times as great. Thus, there is close agreement between the comparative combining power of antiserum 9 W against homologous and heterologous enzyme, whether determined as antibody neutralization titre or calculated from the graphically determined equivalence point. Precipitation tests showed that only when 6346 penicillinase in excess of the equivalent amount was added to 749 antiserum did penicillinase activity appear in the supernatant fluid (Table 2).

The neutralization curve obtained by treating 749 and 6346 penicillinases with antiserum 9 S were similar in form to those obtained with antiserum 9 W, but showed approximately twice the residual activity.

Enzyme neutralization with 6346 *antisera.* The 6346 antisera neutralized the homologous enzyme in one case (antiserum 6 A) very weakly and in the other (antiserum 6 B) not at all (Fig. 3). Surprisingly, however, the 6346 antisera had greater neutralizing power for the 749 penicillinase than for the homologous enzyme (Fig. 3), the neutralization titre being 3·3 times as great (serum 6 A). Possible reasons for this finding are discussed below.

Other cross-reactions. The effects of the above antisera and of the antiserum to purified *Bacillus cereus* 569 penicillinase on the action of *B. subtilis* and *B. cereus*

Table 2. *Precipitation of* Bacillus subtilis *penicillinase activity by antisera to* 749 *and* 6346 *penicillinase*

Enzyme and antiserum were mixed in a final volume of 3·0 ml., containing 0·22 ml. 2 M-NaCl, 0·01 M-phosphate buffer, pH 7·0, and $3 \cdot 3 \times 10^{-4}$ M-oxine and incubated overnight (16–20 hr.) at 35°. After centrifuging down precipitates and removing supernatant fluids, precipitates were washed once and then resuspended in buffered saline. Both original supernatant fluid and washed precipitate were analysed for penicillinase activity. In some experiments, residual neutralizing activity of the supernatant fluid was measured. The adsorbed antisera had first been incubated overnight at 35° with 4 mg./ml. (serum 9 W) or 1 mg./ml. (serum 6 A and 6 B) of the appropriate uninduced preparation. These preparations contained 20 units/mg. (749) or 4·4 units/mg. (6346) basal penicillinase activity. The precipitates which formed were removed by centrifuging and the sera used as shown. The figures given are corrected for CO_2 retention by the buffered saline.

Expt.	Antiserum	Ml. antiserum added	Enzyme	Units added	Neutralizing antibody remaining in supernatant (%)	Percent penicillinase activity in	
						Supernatant	Precipitate
1	749 (9 W)	0·1	749	95	—	4	11
				189	—	6	21
				378	—	62	2
2	749 (9 W)	0·2	749	111	30	4	13
				221	15	4	22
				332	0	12	27
3	749 (9 W)	0·5	749	357	45	< 1	10
				535	28	< 1	14
				713	14	2	18
				892	1	6	19
				1070	2	30	12
4	749 (9 W)	0·2	6346	16	—	6	10
				33	—	5	17
				49	—	3	20
				52	—	0	23
				80	—	22	8
				120	—	41	2
5	6346 (6 A)	0·1	6346	37	—	86	6
		0·2		37	—	89	5
		0·3		37	—	75	6
		0·5		37	—	21	42
6	6346 (6 A)	1·0	6346	34	—	8	54
		2·0		34	—	5	29
7	6346 (6 B)	0·5	6346	34	—	23	75
		1·0		34	—	29	78
		2·0		34	—	46	63
8	6346 (6 B)	1·0	6346	34	—	46	61
		2·0		34	—	83	30
9	6346 (6 A)	0·1	749	37	—	65	31
		0·2		37	—	18	49
		0·3		37	—	18	34
		0·5		37	—	10	7
		1·0		37	—	9	3
		2·0		37	—	2	2
10	6346 (6 B)	0·2	749	38	—	62	28
		0·5		38	—	85	11
		1·0		38	—	63	0
		2·0		38	—	63	0

Table 2 (*cont.*)

Expt.	Antiserum	Ml. antiserum added	Enzyme	Units added	Neutralizing antibody remaining in supernatant (%)	Percent penicillinase activity in	
						Supernatant	Precipitate
11	749 (9W)	0·35	749	545	—	4	19
	749 (9W) (adsorbed)*	0·35	749	545	—	9	18
12	6346 (6A) (adsorbed)*	0·44	6346	34	—	75	16
		0·88		34	—	0	62
		1·8		34	—	3	45
	6346 (6B) (adsorbed)*	0·44	6346	34	—	31	69
		0·88		34	—	53	54
		1·8		34	—	76	28

* Neutralization tests showed that adsorption by uninduced preparations caused a 20 % reduction in neutralizing power of serum 6A and no measurable reduction in that of serum 9W.

enzymes are summarized in Table 1. It is seen that there was no cross reaction, as measured by enzyme neutralization, between the penicillinases of the two bacterial species.

The effect of uninduced culture supernatant fluids on the neutralizing power of antisera

Since impure enzyme preparations were used throughout this work, the possibility arose that substances other than the enzyme itself might be influencing the reaction between enzyme and antiserum. This was investigated by measuring the effect of material from uninduced cultures on the neutralization reactions. Such material is thought to be the same as that found in an induced culture, lacking only induced penicillinase. Addition to the 6346 or 749 enzyme preparation of an equal or greater weight of the corresponding uninduced culture preparation did not affect the neutralization of either enzyme by homologous or heterologous antisera (Fig. 4).

Precipitation of penicillinase activity with antisera

When the *Bacillus subtilis* enzyme preparations were mixed with antisera and incubated overnight at 35°, precipitates possessing different degrees of penicillinase activity were formed. No precipitates were formed with normal rabbit serum. The results of representative experiments, in which different proportions of homologous and heterologous antisera were mixed with 749 and 6346 penicillinase, are presented in Table 2. The following were the main findings.

(1) With 749 penicillinase and its antiserum (9W), the activity of the precipitate formed varied with changing enzyme-antibody ratio. It was approximately 10 % in the presence of large antibody excess, corresponding to the residual activity found in neutralization curves (Fig. 1), and rose to 20 % or more at the equivalence point (determined by the simultaneous disappearance of antibody from, and appearance of antigen in, the supernatant). As the proportion of enzyme was further increased, less activity was found in the precipitate; virtually no activity was found in precipitates formed in the presence of a large excess of penicillinase. This failure of a precipitate to form in the presence of large antigen excess is characteristic of

other antigen–antibody combinations (Boyd, 1956). Antiserum 9S gave results similar to those obtained with serum 9W. The active precipitate formed by the combination of 749 penicillinase with antiserum 9W proved, as did the precipitate formed between *Bacillus cereus* 569 penicillinase and its antiserum (Pollock, 1956*a*), quite stable to repeated washing in buffered saline.

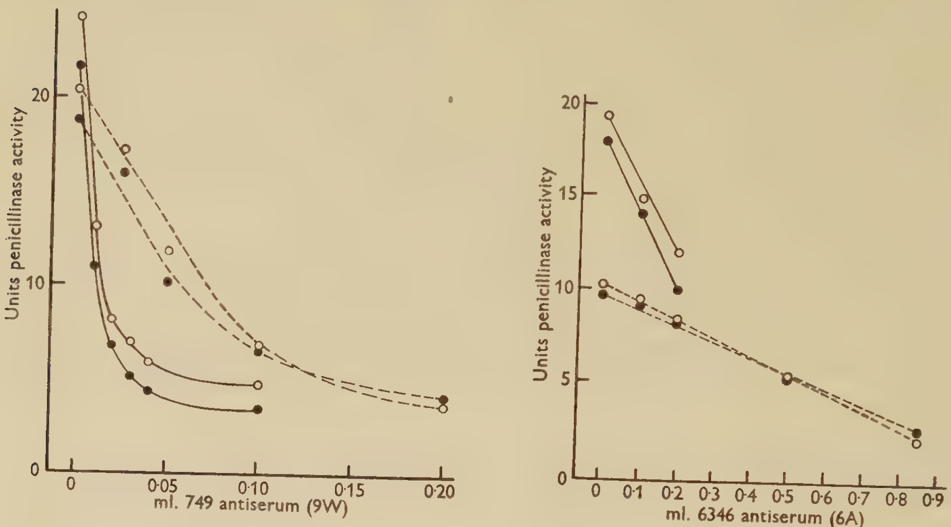

Fig. 4*a*

Fig. 4*b*

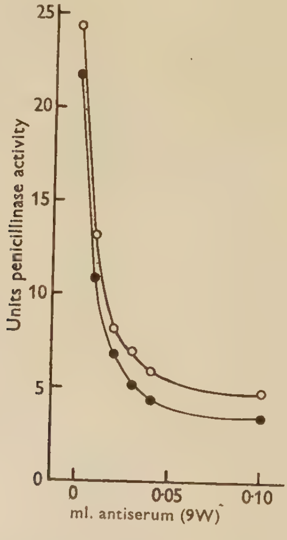

Fig. 5

Figs. 4*a*, *b*. Effect of preparations from uninduced cultures on neutralization of penicillinase by 749 antiserum 9W (4*a*) and 6346 antiserum 6A (4*b*): 749 penicillinase (0·05 mg) ●—●; 749 penicillinase + uninduced 749 preparation (0·05 mg.) ○—○; 6346 penicillinase (0·3 mg. in 4*a*; 0·15 mg. in 4*b*) ●---●; 6346 penicillinase + uninduced 6346 preparation (0·5 mg. in 4*a*; 0·2 mg. in 4*b*) ○---○.

Fig. 5. Neutralization of basal and induced 749 penicillinase by 749 antiserum 9W. Induced penicillinase (0·06 mg.) ●—●. Basal penicillinase (1·0 mg.) ○—○.

(2) Precipitates having penicillinase activity were also formed between the 6346 enzyme preparation and the 749 antiserum 9 W. The activity of these precipitates varied with changing enzyme: antibody ratio in a manner similar to that of the precipitates formed between 749 penicillinase and antiserum 9 W.

(3) As already noted (Fig. 3), 6346 antisera had a greater inhibitory action on 749 penicillinase than on 6346 penicillinase. It was also found that serum 6 A had a greater precipitating power with the heterologous than with the homologous enzyme. For example, 0·2 ml. of this serum removed 30 units of 749 penicillinase activity from the supernatant fluid but only 4 units of 6346 penicillinase activity (Table 2; Expts. 5 and 9). The actual amount of enzyme precipitated cannot be determined from these experiments, but it is evident that not more than 4 units of 6346 penicillinase and not less than 18 units of 749 penicillinase were precipitated by 0·2 ml. serum 6 A. Thus, the difference in combining power of antiserum 6 A with the heterologous and homologous enzymes was slightly larger than the respective difference in neutralization titre (Fig. 3). Certain precipitates formed in the presence of serum 6 A had much more penicillinase activity than precipitates formed in the presence of 749 antisera, some containing more than 50 % original activity. With increasing amounts of antiserum, the activity of these precipitates decreased.

(4) Striking, though not easily interpretable, results were obtained with antiserum 6 B. This serum had previously been found to have no inhibitory action on 6346 penicillinase and only a slight action on 749 penicillinase (Fig. 3). The lack of inhibition of the homologous enzyme, however, was not due to a lack of combining power, but to the fact that antibody in this serum combined with the enzyme without lowering its activity. Thus, precipitates with almost 80 % original enzymic activity could be obtained, the remainder of the penicillinase activity remaining in solution. With increasing amounts of antiserum, however, less activity was precipitated; because of this, it seemed difficult to estimate the combining power of this serum. Significant amounts of 749 penicillinase activity could be precipitated by serum 6 B, but with increasing amounts of serum, either the precipitate formed was inactive, or no enzyme was precipitated. Since the neutralizing power of serum 6 B was low and its precipitation reactions obscure, further work was not done with this serum.

(5) The penicillinase preparations used had not been purified, and the antisera must have contained antibodies other than those to penicillinase. An attempt was made to purify the antisera, at least partially, by incubating them overnight with material from the appropriate uninduced culture supernatant fluid and removing the large precipitate that formed. These adsorbed antisera caused precipitates to form with approximately the same penicillinase activity as those formed in experiments with corresponding amounts of untreated antisera (Expts. 11 and 12).

Immunological comparison of basal and induced 749 *penicillinase*

The uninduced 749 culture had a measurable basal penicillinase activity, approximately one-fifteenth that of the induced culture. The neutralization curve of the basal enzyme by 749 antiserum was parallel throughout its course with that of the induced enzyme (Fig. 5), indicating that, as with *Bacillus cereus* 569 (Pollock, 1956*a*), basal and induced enzymes are immunologically indistinguishable.

DISCUSSION

There is no obvious explanation for the curious fact that the antiserum from rabbit A, the most active of the two 6346 antisera obtained, both precipitated and neutralized the heterologous enzyme more effectively than it did the homologous enzyme. The combining power of 6346 penicillinase, even with its own antiserum, thus seems to be lower than that of the 749 penicillinase, unless it is possible that in these experiments combination did not necessarily lead to precipitation, or unless one makes the assumption that the specific activity of 6346 penicillinase is much lower than that of 749 penicillinase. In the latter case, combination with more molecules of 6346 penicillinase than of 749 penicillinase would be necessary for the same degree of enzyme inhibition. Such a large difference in specific activity between similar enzymes from closely related species would appear unusual (Dixon & Webb, 1958, p. 653), but certainly not impossible, since catalytically similar enzymes with different specific activities may be formed by the *same* species (Brown, 1952; Neilands, 1952). Thus, ox liver has been reported to contain two catalases possessing a fourfold difference in specific activity (Brown, 1952). It should be noted, however, that the difference in specific activity (on a molecular basis) between the immunologically distinct penicillinases of *Bacillus cereus* 569 and *B. cereus* 5/B is only 20% (Kogut *et al.* 1956).

If differences in combining power of the two *Bacillus subtilis* penicillinases do, in fact, account for the anomalous cross-reactions observed, the following possibility might be considered. It is reasonable to suppose that the antigenically active groups shared by the two types of penicillinases could differ somewhat in their ability to combine with the antibody against these groups. This difference might be such that the antigenically active groups on the 749 enzyme were more 'exposed' (or for other reasons more reactive) than those on the 6346 penicillinase, and possibly the combination of antibody with such groups would inhibit enzyme activity more effectively. In such a case the 749 enzyme might combine more effectively with a higher proportion of the specific antibody (even when induced by the heterologous 6346 penicillinase) than could the 6346 penicillinase itself. There seems to be no theoretical objection to such an apparently paradoxical situation arising occasionally; although it might be expected to be infrequent. To determine precisely what differences between the two penicillinases might account for their anomalous cross-reactions, it would be necessary to have both enzymes in a pure form.

The fact that purified enzymes were not available for this work has made it necessary to view with caution some of the results, especially those showing that precipitates possessing a wide range of penicillinase activity may be formed between different combinations of *Bacillus subtilis* penicillinases with their antisera. Since the rabbits had been injected with a mixture of proteins, their sera must have contained antibodies other than those against penicillinase, and it seemed possible that formation of precipitates with penicillinase activity might be due to a non-specific co-precipitation of penicillinase by other antigen + antibody pairs present rather than to penicillinase + antipenicillinase interaction. Although this possibility has not been rigidly excluded, it appears highly unlikely for the following reasons.

(1) With an increasing antigen: antibody ratio, 749 penicillinase activity appears in the supernatant fluid at the same time as neutralizing antibody disappears

(Table 2). A close correspondence between the two events, characteristic of antigen + antibody combination, would not be expected if removal of enzymic activity were chiefly by co-precipitation.

(2) Antisera adsorbed with uninduced culture preparations, in order to remove antibodies other than antipenicillinases, formed precipitates possessing approximately as much penicillinase activity as those formed with untreated antisera (Table 2).

(3) *Bacillus subtilis* penicillinases were not carried down by the precipitates formed between defined antigen + antibody pairs. It was found that the precipitation of Pneumococcal Type III polysaccharide, of human gamma globulin, and of ovalbumin by their specific (rabbit) antisera (generously given by Dr J. H. Humphrey and Dr B. A. Askonas) in the presence of 6346 or 749 penicillinase did not remove any penicillinase activity from solution.

For these reasons, the enzymic activity of the precipitates formed when penicillinase preparations were mixed with antisera is considered a measure of the activity of the enzyme + antibody complex. The formation of such an active complex is of special interest in considering the inhibitory pattern of the 6346 antisera. Rabbits injected with 6346 penicillinase over a prolonged period gave discouragingly low titres of antibody activity, as measured by 6346 penicillinase neutralization (rabbit A), or had no neutralizing ability at all (rabbit B). Precipitation tests showed, however, that the reaction between 6346 antisera and the homologous enzyme was greater than indicated by enzyme inhibition alone, and that combination with antibody (as indicated by precipitation) could take place with little or no accompanying inactivation. A considerable difference in enzymic activity of penicillinase + antipenicillinase precipitates in two *Bacillus cereus* strains was observed earlier (Pollock, 1956*a*); strain 569 penicillinase formed a precipitate with less than 5% original activity, whereas strain 5/B penicillinase formed a precipitate with over 50% original activity, with the homologous antiserum.

Cinader (1957) emphasized that combination of an enzyme with its antibody may or may not lead to inactivation and that, in general, active precipitates may be formed only with those enzymes possessing low molecular weight substrates. The studies reported here and the earlier work of Pollock (1956*a*) have emphasized how the degree of inactivation can also depend on the properties of the enzyme and antibody involved. Depending on the bacterial strain producing the enzyme and the antiserum used, one may obtain a series of enzyme + antibody precipitates in which enzyme activity is absent, or is almost completely unimpaired.

I am deeply indebted to Dr M. R. Pollock for first suggesting this problem and for many discussions during the course of the work; to Dr J. H. Humphrey for invaluable advice on immunological techniques and for discussing the results obtained. Miss Ruth Coyle and Mr Richard Pearce provided expert technical assistance. Experimental studies were carried out through a work transfer under the Research Branch, Canada Department of Agriculture, from the Cytogenetics Section, Forest Insect Laboratory, Sault Ste. Marie, Ontario, Canada.

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Inter-relationships of Alleles at the *am* Locus in *Neurospora crassa*

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SUMMARY

It has been shown that the alleles 5, 6 and 7 at the *am* locus in *Neurospora crassa* backmutate; the alleles 4, 8, 9 and 11 do not backmutate at detectable frequencies. Wild-types were recovered in low frequencies from crosses between different *am* alleles. It has been shown that each of the twelve *am* alleles, so far investigated, is unique with respect to at least one of the following criteria: mutation, recombination, complementation, type of glutamic dehydrogenase produced. It is argued that the minimum number of possible mutations at the *am* locus, which can affect glutamic dehydrogenase production, is eighteen. The probability that this minimum estimate is too large is less than 1 %.

INTRODUCTION

It is known that mutations at the 'amination deficient', *am*, locus in *Neurospora crassa* specifically affect the formation of the enzyme glutamic dehydrogenase by the organism (Fincham, 1954; Pateman & Fincham, 1958). A number of independently induced *am* mutants have recently become available. This paper is chiefly concerned with the inter-relationships between ten independently induced *am* mutants as determined by the criteria of complementation, mutation and recombination.

METHODS

Experimental strains. The origin of the *am* strains and of the stock wild types was described previously (Pateman & Fincham, 1958; Fincham, 1959). The symbols used for the *am* mutants in the present paper differ from those used by Pateman & Fincham (1958). The superscripts, 32, 47, 29 and R, are replaced and all the *am* strains are now specified by a simple numerical superscript forming the series am^1 to am^{11}. For the sake of clarity, *am* strains will usually be referred to by number only. For example, am^2 would be denoted by 2, the locus symbol *am* being understood.

Culture media. For growth tests and stock cultures Fries No. 3 minimal medium was used, with suitable supplements where necessary. In the backmutation experiments the usual energy source, 1·5 % (w/v) sucrose, was replaced by 1·5 % sorbose +0·75 % sucrose. On this media *Neurospora crassa* has a colonial growth habit. All crosses between strains were made at 25° on a medium favouring sexual reproduction (Westergaard & Mitchell, 1947).

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Experimental procedure in irradiation experiments. The details of the backmutation experiments were similar to those previously described by Pateman (1957), with the following exceptions. The source of ultraviolet radiation was a General Electric Co. (G-15 T-8), 15 w low-pressure mercury-vapour germicidal lamp. During irradiation the conidial suspension formed a layer approximately 3·0 mm. thick in an open Petri dish. The surface of the suspension was approximately 8·0 cm. from the lamp; under these conditions an exposure time of 90 sec. killed 99·0 % of the conidia.

RESULTS

Backmutation of am *alleles*

It was shown previously (Pateman, 1957) that the *am* strains 1, 2 and 3 will backmutate as a result of ultraviolet irradiation. Similar series of backmutation experiments were carried out with strains 4, 5, 6, 7, 8, 9 and 11. Macroconidia were collected from 14-day cultures grown at 25°, then irradiated for 90 sec. to give a *c.* 99 % kill, and spread on Fries medium + sorbose + glycine agar contained in Petri dishes. The survival rate of conidia after irradiation varied from 0·5 to 4 % in different experiments, but in the majority was close to 1 %. The number of live *am* conidia after irradiation in each experiment was usually between $0{\cdot}5 \times 10^6$ and $1{\cdot}5 \times 10^6$. The total number of backmutation experiments with the seven *am* alleles was thirty-eight. The results of these experiments are given in Table 1. After incubation at 25° for 72–90 hr. any wild-type colonies which appeared were transferred to minimal medium slopes. The resultant cultures were subsequently tested for growth on minimal medium to check their apparent wild phenotype.

Table 1. *Frequency of backmutation in certain* am *alleles in* Neurospora crassa

am allele	Total no. of live conidia ($\times 10^{-6}$)	Total no. of wild types	Mean backmutation rate per 10^6 live conidia
4	11·71	1	—
5	1·29	26	*c.* 20·0
6	1·40	37	*c.* 26·4
7	10·80	21	*c.* 1·9
8	4·86	0	—
9	8·97	0	—
11	7·71	0	—

It is clear from Table 1 that the *am* alleles fell into two distinct groups. One group contained 4, 8, 9 and 11, all of which did not backmutate at a detectable frequency. One wild-type colony was in fact recovered from experiments with 4. This might have been a contaminant, but if genuine, then the backmutation frequency of 4 was certainly much lower than that of the 5, 6 and 7 alleles. The other group comprised 5, 6 and 7 which did backmutate at frequencies sufficiently high to be easily detected. The approximate backmutation frequencies obtained for 5 and 6 are not significantly different. The frequency found for 7 is significantly different from both 5 and 6; comparison of 7 with 5 and 6 combined gives a value of $t_{10} = 7{\cdot}621$, $P < 0{\cdot}001$.

The characteristic backmutation frequency of each *am* allele might be a specific property of the allele itself or, at least in part, a reflexion of the background genotype. Each of the *am* strains used in these experiments had been backcrossed at least three and usually four or more times to the same stock strain, St Lawrence wild type (Pateman, 1957). Therefore the stocks were largely isogenic and it is probable that the large difference in backmutation frequencies between the two groups of alleles was due to specific characteristics of the alleles themselves. The significant difference in backmutation frequency between 5 and 6 and the other allele in that group (7) also probably reflects different specific properties of the actual alleles. There are two main points established by these experiments. The *am* alleles 5, 6 and 7 are sharply distinguished from 4, 8, 9 and 11 by the criterion of backmutation. The genetic change which results in the manifestation of the *am* phenotype in 5, 6 and 7 cannot be a deletion, since these strains readily backmutate.

Recombination of am *alleles*

It is known (Pateman, 1958, 1960) that true wild types can be recovered from crosses between am^2 and am^3 with a frequency of about 1/50,000 live ascospores. A further series of experiments in which crosses between different *am* alleles were analysed with respect to the production of wild types is described here. The experimental details are similar to those previously described by Pateman (1958) and consisted essentially of plating ascospores from *am* × *am* crosses on a glycine medium on which the am^+ ascospores had a strong selective advantage. Apparent am^+ ascospores were isolated, cultured and subsequently tested in growth flasks to confirm that they were phenotypically wild-type. The results of these experiments are given in Table 2; the results given in line 1 are from Pateman & Fincham (1958) and Pateman (1960). It has been shown (Pateman & Fincham, 1958) that wild-types are not recovered from crosses between identical *am* parents. Therefore the data in Table 2 show that recombination occurred between the pairs of *am* strains listed, with the exception of 5 and 7. In the latter case recombination was doubtful since only one wild type was recovered.

Table 2. *Recovery of wild-types from* am × am *crosses*

Cross	No. of wild types	No. of live ascospores (thousands)	Frequency of wild types per 10^6 live ascospores
2 × 3	104	5443	19·1
4 × 8	8	513	16·0
4 × 9	22	305	72·0
4 × 11	14	164	85·0
5 × 6	9	715	13·0
5 × 7	1	1355	0·75
6 × 7	9	586	15·0
8 × 9	4	566	7·1
8 × 11	25	504	50·0
9 × 11	3	712	4·2

Differentiation of am *alleles*

There are four criteria available for differentiating between the known *am* alleles, namely, mutation, recombination, complementation, and type of enzyme produced.

Mutation and recombination have been used in previous sections. It is necessary at this point to outline the situation with regard to complementation. It has been shown (Fincham & Pateman, 1957*a*; Pateman & Fincham, 1958; Fincham, 1959) that certain combinations of *am* alleles in heterocaryons were complementary with respect to the production of glutamic dehydrogenase. The only combinations of alleles from the present series which were complementary were 1+2 and 1+3. Either of these pairs of alleles gave heterocaryons which grew on minimal medium without lag and yielded detectable glutamic dehydrogenase activities up to about 25 % of that normally found in wild-type.

In addition to the *am* alleles which result in a complete lack of detectable glutamic dehydrogenase activity, a number of secondary mutant alleles are known (Pateman, 1957). These are backmutations at the *am* locus which restore some glutamic dehydrogenase activity, but not the normal amount found in wild-type strains. Two of these unusual backmutants have been further investigated (Fincham, 1957; Fincham & Pateman 1957*b*; P. Bond, unpublished; J. R. S. Fincham, unpublished) and shown to possess a qualitatively different glutamic dehydrogenase both from wild-type and from each other. So far, only one of these strains, designated am^{2l}, has been tested for complementation with the other alleles (Fincham, 1959).

Table 3. *Relationships between* am *alleles in* Neurospora crassa

	Alleles which result in the production of a qualitatively different glutamic dehydrogenase	Combinations of alleles which show complementation	Alleles which backmutate	Alleles which do not backmutate or show complementation
	21 1038*	1+2, 1+3 1+2*l*, 3+2*l*, 4+2*l*, 6+2*l*, 7+2*l*, 8+2*l*, 9+2*l*, 11+2*l*	1, 2, 3 5, 6, 7	4, 8, 9, 11 —
Pairs of alleles which show recombination	—	2×3	2×3, 5×6, 5×7?, 6×7	4×8, 4×9, 4×11, 8×9, 8×11, 9×11

* 1038 is the isolation number; 689 is the isolation number of am^{21} (Pateman, 1957).

Table 3 is a summary of all the relevant information concerning the *am* alleles arranged to illustrate the point that each of the twelve so far investigated is unique. The alleles 2*l* and 1038 are clearly distinct from each other and all the rest since they result in the production of qualitatively different enzymes. Within the series 1–11 each allele can be shown to be unique as follows. Only 1 is complementary with 2 and 3 and is therefore distinct from all the rest. Since only 2 and 3 are complementary with 1 they form a separate group, but 2 and 3 are distinct since they show recombination. The alleles 1, 2, 3, 5, 6 and 7 form a separate group since they backmutate; within this group recombination indicates that each allele is unique with the possible exception of 5 and 7. Since only one wild-type was recovered from the cross 5 × 7, recombination is doubtful. But 7 is complementary with 21 and 5 is not, also 7 gave a significantly different backmutation frequency from 5, therefore 5 and 7 are different. The alleles 4, 8, 9 and 11 form a separate group since they do not backmutate at a detectable frequency. Recombination shows that each member of this group is distinct from the rest. Thus it is clear that each of the twelve alleles is different from the rest with respect to at least one of the four criteria which can be used to differentiate between them.

Number of possible kinds of mutation at the am locus

It is of interest to attempt to determine an estimate of the minimum number of sites which can be altered by mutation in a complex locus. Such estimates have been derived for adenine loci in Saccharomyces (Roman, 1956), for histidine loci in Salmonella (Hartman, Hartman & Šerman, 1960) and for complex loci in Drosophila, Aspergillus, bacteriophage and Schizosaccharomyces (Pontecorvo, 1958). In all these cases the method of estimation depends essentially on recombination data. In the case of the *am* locus in *Neurospora crassa* the situation is somewhat different. The first twelve alleles investigated were derived from five independent sources, ten were induced by ultraviolet radiation, one by 20-methylcholanthrene and one by β-propiolactone. Each of the twelve is unique as judged by four independent criteria, of which recombination is one. If it be assumed that they approach a random sample, then an estimate of the minimum number of possible mutations at the *am* locus which can affect glutamic dehydrogenase production may be made. It should be noted that the estimate concerns the minimum number of different kinds of mutation at the *am* locus which can affect glutamic dehydrogenase production, not the minimum number of mutational sites. It is possible that more than one kind of mutation can occur at a mutational site within the locus. The minimum estimates made by other workers, referred to above, concern mutational sites separable by recombination, since the estimates were essentially based on recombination data only.

The argument on which the estimate of the minimum possible number of *am* alleles rests is as follows. Assume that the possible kinds of mutation at the *am* locus are equally likely. The question of estimation then concerns the probable size of the population of different, equally likely, kinds of mutation from which a random sample of twelve prove to be all different. It can readily be shown, if a random sample of size r is taken from a population made up of n kinds of event, that P, the probability all r will be different is given by

$$P = \frac{n!}{n^r(n-r)!}.$$

In the present case r is the number of different alleles, n the number of different kinds of mutation. For $r = 12$, $n = 26$, $P = 0{\cdot}04843$. For $r = 12$, $n = 18$, $P = 0{\cdot}007679$. The latter set of conditions is equivalent to the following statement. Since each of the first twelve alleles investigated is unique, there are at least eighteen different possible kinds of mutation at the *am* locus, and the probability that this estimate is too large is less than 1 %. Similarly it can be stated that the minimum number of possible kinds of mutation is twenty-six, with a probability that this estimate is too large of less than 5 %. The estimates are minimum ones for the appropriate probability levels. The actual number of possible kinds of mutation may be higher.

It is possible, indeed probable, that the assumption that all the possible kinds of mutation are equally likely, is incorrect. This would mean that one (or more) of the possible kinds of mutation was more frequent than the rest. Such a relatively frequent mutation would stand a proportionally greater chance of being included twice in the random sample, whatever the size of the latter. Consequently, the existence of a relatively frequent mutation can only decrease the average size of the sample which contains only non-identical mutations. That is, the existence of one or more relatively frequent mutations could only decrease the estimate of the minimum number of possible kinds of mutation. Thus the possibility of different relative mutation frequencies does not invalidate the estimates of the minimum number of possible kinds of mutation at the *am* locus.

DISCUSSION

It is now known that in many respects the *am* locus in *Neurospora crassa* is similar to other complex loci which have been investigated in detail. Recombination between *am* alleles, at least partly due to a mechanism other than classical crossing-over (Pateman, 1958, 1960), complementation, and differences in mutation frequency, have all been demonstrated. These are phenomena commonly associated with complex loci in bacteriophage, bacteria, fungi and higher organisms. The phenomenon of complementation represents a functional aspect of the complex locus. Benzer (1957) proposed the term 'cistron' for the functional unit of the gene. The definition of the cistron is an operational one based on complementation. Several cases are already known which do not fit the cistron concept in its simplest form. The *ad*-9 locus in Aspergillus (Calef, 1957) and the *hisB* locus in Salmonella (Demerec & Hartman, 1959) are examples. In the case of the *am* locus in Neurospora, neither the whole locus nor any subunit corresponds to a cistron. The fact that certain combinations of alleles show complementation implies the existence of functional subunits within the *am* locus. But most of the possible combinations of *am* alleles are non-complementary. Consequently to fit the cistron concept, the majority of the apparent alleles would need to be regarded as deletions of various sizes (Fincham, 1959). Such a numerical preponderance of deletions over point mutations as a result of ultraviolet irradiation seems unlikely. Also 2, 5, 6 and 7, each of which would cover two or more cistrons, are known to backmutate and thus cannot be deficiencies.

In view of this consideration there appear to be two main possibilities concerning the situation at the *am* locus in Neurospora. Either the complementary relationships between the alleles represent an aspect of the *am* locus which is non-linear, or some of the point mutations occur at sites especially critical for the production of an integrated gene product, in this case presumably the enzyme glutamic dehydrogenase. The latter alternative seems, to the author, the most probable. It is similar to a possibility suggested by Demerec & Hartman (1959) in connexion with a number of alleles, which are at variance with the cistron concept, in the *hisB* locus in *Salmonella typhimurium*.

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CORRIGENDA

In Perrett, C. J. (1960). *J. gen. Microbiol.* 22, 589–617.

On p. 589, line 9 of Summary:
for 'time-dependent' *read* 'time-independent'.

On p. 605, 21 lines from bottom:
for 'system cannot only' *read* 'system can not only'.

On p. 608, 5 lines from bottom:
for 'large' *read* 'dense'.

In Gebicki, J. M. & James, A. M. (1960). *J. gen. Microbiol.* 23, 9.

On p. 10, line 23:
for *Aerobacter aerogenes* (NCTC, no. 240)
read *Aerobacter aerogenes* (NCTC, no. 418)

In Wittler, R. G., Malizia, W. F., Kramer, P. E., Tuckett, J. D., Pritchard, H. N. & Baker, H. J. (1960), *J. gen. Microbiol.* 23, 315.

On p. 327, Table 2, *under* Strain 6–4a, Variants 1 and 2:
for 'Date of test—May 1959' *read* 'Date of test—July 1959'

ADDENDA

On p. 331, before Acknowledgements add:

The Corynebacterium strains referred to in this paper have been deposited in the American Type Culture Collection, 2112 M Street, N.W., Washington 7, D.C., U.S.A., and have been assigned the following numbers:

Corynebacterium (*acnes?*) variant 52–3 = ATCC no. 13959
,, ,, ,, 6–4a–3 = ATCC no. 13960
,, ,, ,, M–5 = ATCC no. 13961
,, ,, ,, 11–4d = ATCC no. 13962

On p. 331. To Acknowledgements add:

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Studies of the Morphological, Physiological, and Biochemical Characters of *Actinomyces bovis*

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SUMMARY

A study was made of 11 bovine strains of Actinomyces. These strains were compared with 15 human strains identified as *Actinomyces israelii* and *Actinomyces naeslundii*. Ten of the bovine strains constituted a homogeneous group identified as *Actinomyces bovis*. One of the bovine strains was identified as *A. israelii*.

The characteristics of *Actinomyces bovis* were the following: it failed to form a true mycelium *in vitro*; it had less tendency than *A. israelii* and *A. naeslundii* to form mycelial elements in lesions in animals into which it was inoculated. Characteristically it formed two types of colonies when first isolated from animals and when maintained in culture. These colonies were: (1) a smooth glistening transparent microcolony having an entire or furred edge; (2) a raised, opaque, rough colony having an entire or furred edge. The microcolonies of *A. bovis* were very characteristic on brain heart infusion agar and were easily differentiated from the mycelial colonies of *A. israelii* and *A. naeslundii*. Variation of the morphology of the colonies occurred rapidly *in vitro* as a result of genetic change and the medium used. When large, on certain media, the colonies of either the rough or smooth variants were sometimes indistinguishable from colonies of *A. israelii* or *A. naeslundii*, but were easily distinguishable on brain heart infusion agar. *A. bovis* was a catalase negative anaerobe which did not hydrolyse gelatin or casein; but in contrast to *A. israelii* and *A. naeslundii* it hydrolysed starch rapidly and completely when tested with $KI + I_2$ solution. *A. bovis* produced acid but no gas from starch but did not form acid or gas from xylose, raffinose, and mannitol, and did not reduce nitrate to nitrite. *A. israelii* did not form acid or gas from starch; it usually produced acid but no gas from xylose and mannitol, and occasionally produced acid but no gas from raffinose; it reduced nitrate to nitrite sometimes. *A. naeslundii* produced no acid or gas from starch; it produced acid but no gas from raffinose but not from xylose or mannitol, and it reduced nitrate to nitrite.

INTRODUCTION

In a comparative study of *Actinomyces* spp. with strains of *Lactobacillus bifidus*, Pine & Howell (1956) did not include bovine strains of *A. bovis* identical with those described by Erikson (1940) or Thompson (1950). Of the two bovine-type strains which had been obtained, one was lost and the second did not give satisfactory growth in the medium used. Repeated attempts to obtain known strains of *A. bovis* from other investigators were unsuccessful. In some cases cultures were obtained but these were either *Corynebacterium* species or cultures of *Actinomyces* identical with *A. israelii*. It was therefore considered possible that the limited acceptance of *A. bovis* as a separate species was due either to the failure of various investigators to recognize strains of *A. bovis* as described by Erikson (1940), or to the possibility that no real species differentiation was warranted. Since the only extensive comparative bacteriological study of *A. bovis* and *A. israelii* was done by Erikson (1940), it appeared to us that re-evaluation of the species *A. bovis* and *A. israelii* was in order. Consequently, 11 strains of Actinomyces from typical cases of lumpy jaw in cows were obtained from other workers or isolated directly (Pine & Watson, 1959). These cultures have now been critically studied in regard to cellular and colonial morphology, physiology and fermentative ability. Comparisons have been made with strains now recognized as *A. israelii* and *A. naeslundii* either directly or on the basis of the results obtained previously (Pine & Howell, 1956; Howell, Murphy, Paul & Stephan, 1959). These investigations, in our opinion, support the taxonomic existence of the species *A. bovis*, as originally proposed by Erikson (1940).

METHODS

Strains of Actinomyces *spp. used*

The strains of Actinomyces isolated from human sources are those given in Table 1. All cultures were maintained on the maintenance medium of Pine & Watson (1959), with the exception of one rough variant of *A. bovis* (*A. bovis* P2R) which was cultured on a 1+1 (v/v) mixture of brain heart infusion agar (Difco) + maintenance medium.

Isolation of single microscopic colonies of Actinomyces bovis

The medium generally used for primary isolations, purification, and studies of cultural variation in the strains of *Actinomyces bovis* was the maintenance medium as a liquid or with 0·7 or 1·5% (w/v) agar. In most cases Garrod's medium and brain heart infusion agar with and without sheep's blood (10%, v/v) were also used for primary isolation as described previously (Pine & Watson, 1959). The composition of Garrod's medium was: beef extract 3·0 g., NaCl 5 g., Bactopeptone 10 g., soluble starch 1·0 g., and 20 g. agar per litre of medium (Prof. L. P. Garrod, personal communication). The incubation temperature used was 37° unless otherwise stated.

To prepare the inoculum for isolation from clinical material, mycelial granules were picked from the pus and transferred to a tube of sterile distilled water. The tube was shaken vigorously, the granules allowed to settle to the bottom, and the supernatant liquid withdrawn with a sterile pipette. This washing procedure was repeated at least twice. After the final washing the granules were ground up in the residual fluid by using a heavy tight-fitting, sterile glass rod. The homogenate was then streaked on the isolation media and the plates incubated in 5% (v/v) CO_2 + 95% (v/v) N_2. The plates, which were examined daily for 3–5 days, were invariably found to be heavily covered with colonies of *Actinomyces bovis*; a few streptococcal colonies were occasionally observed. However, as repeated isolations were made from the same sample of frozen pus (Pine & Watson, 1959) greater numbers of contaminating colonies were obtained. To purify or to pick variant

Table 1. Actinomyces *spp. used*

Strain no.	Source	Donor
	Human strains	
699	Lacrimal canaliculitis	Duke University Medical School, Mycology Section, Durham
OS131	Lacrimal canaliculitis	Duke University Medical School, Mycology Section, Durham
843	Actinomycosis of the upper jaw	Duke University Medical School, Mycology Section, Durham
534	Actinomycosis of the elbow	Duke University Medical School, Mycology Section, Durham
895	Thoracic actinomycosis	Duke University Medical School, Mycology Section, Durham
261	Gingival scrapings	Dr Ada Clark and Dr Solon A. Ellison, Department of Bacteriology, College of Physicians and Surgeons, Columbia University
262	Cervico-facial actinomycosis	Dr Ada Clark and Dr Solon A. Ellison, Department of Bacteriology, College of Physicians and Surgeons, Columbia University
263	Cervico-facial actinomycosis	Dr Ada Clark and Dr Solon A. Ellison, Department of Bacteriology, College of Physicians and Surgeons, Columbia University
282	Mandibular abscess	Dr Per Holm, State Serum Institute, Copenhagen
283	Mandibular abscess	Dr Per Holm, State Serum Institute, Copenhagen
286	Gingival scrapings	Dr Robert J. Fitzgerald, National Institute of Dental Research, Bethesda
287	Cervico-facial actinomycosis	Dr Solon A. Ellison, Department of Bacteriology, College of Physicians and Surgeons, Columbia University
279	Sinus following tooth extraction	Dr Luther Thompson, Section on Bacteriology, Mayo Clinic, Rochester, Minn.
295	Human pleural fluid	ATCC 10048
296	Atypical actinomycosis of the ankle	ATCC 10049
	Animal strains	
P1S; P2S, P3S, P4S and variants; lumpy jaw in cows		Isolated in present work
A1, A8, A9, A10, A11, A12; lumpy jaw in cows		Dr Libero Ajello and Dr William Kaplan, U.S.P.H. Communicable Disease Center, Chamblee, Ga.
A601.	Lumpy jaw in cow	Dr Esther Meyer, Department of Bacteriology, College of Medicine, University of Illinois, Chicago, Ill.

colonies of pure cultures, the inoculum was streaked on agar plates and incubated for 24–48 hr. The plates were then examined with the scanning objective of the microscope (total magnification, × 75). When a colony was chosen for transfer, it was centred in the microscopic field, and the objective was swung to low power (× 150). The diaphragm of the light source was decreased until the diameter of the circle of light was only slightly larger than that of the microcolony. The substage condenser was focused so that Koehler illumination resulted, i.e. the image of the light source was in focus on the surface of the agar plate. The objective was raised from the plate and, keeping the plate in place, the circle of light was outlined at its circumference by stabbing the agar successively with a stiff inoculating needle which had been sharpened to a fine point and bent at a 90° angle. The colony was then re-examined under the scanning objective to ascertain that only the desired colony was circumscribed and that no neighbouring colonies had been touched. The plate was removed from the microscope stage and the small disk of agar carrying the colony was picked from the plate and transferred to a tube of maintenance liquid medium. The plate was re-examined to make certain that neighbouring colonies had not been disturbed in picking the agar disk.

The microcolonies of *Actinomyces* spp. adhered to the agar disks tenaciously. The disks were observed daily at the bottom of the tube of broth by using a hand lens and the original colonies generally were seen to grow into single large colonies which separated from the agar with shaking. When such disks showed multiple colonies or when they showed growth emanating from other points of the agar disk, they were discarded. In primary isolation from infected cow heads, cultures from single colonies were restreaked and single colonies picked again. This was repeated a third time before stock cultures were made.

General cultural and chemical methods. The procedures for the growth and preparation of inocula for all the cultures and measurements of growth were those described previously (Pine & Howell, 1956). To make direct comparisons with the human strains of Actinomyces (Pine & Howell, 1956) all physiological studies were made by using the liquid defined medium + starch + Casitone (Difco) of Pine & Howell (1956), or in some cases by using the maintenance medium. However, cultural and morphological studies were also done by using Garrod's medium and brain heart infusion agar (Difco), with and without 10 % (v/v) blood; fluid thioglycollate (Baltimore Biological Laboratory), and thioglycollate agar. The anaerobic and aerobic seals used, with or without carbon dioxide, and all chemical, physiological and fermentation methods were as described previously (Pine & Howell, 1956). All plates were incubated in an atmosphere of 5 % (v/v) CO_2 + 95 % (v/v) N_2.

All strains were tested for pathogenicity in hamsters (Hazen & Little, 1958). Cultures for animal inoculation were grown in 5 ml. maintenance medium for 48–72 hr. The organism was centrifuged and the sediment homogenized in 5 ml. sterile distilled water. Each animal, aged 4–6 weeks, was inoculated with the homogenized suspension intraperitoneally with 0·5 ml. and intradermally on each side of the abdomen with 0·2 ml. The inoculated animals were observed for 10–30 days. During this time, abscesses which formed in the skin were drained by first rubbing the surface of the skin with ethanolic iodine solution and then puncturing with a needle. Gram stains of the pus were made and examined for the presence of typical actinomycotic mycelial elements. Such lesions were drained on successive occasions until they healed spontaneously or until the animal was sacrificed. Generally the abscesses formed a 'head' by the 5th to 7th day, ruptured, and healed by the 10th day. In the experiment presented in Table 3, animals were inoculated intraperitoneally and intradermally and re-inoculated 7 days later. All animals were then sacrificed 30 days after the first inoculation. Other methods or procedures when used are given in the text.

RESULTS

Morphological and cultural characteristics of bovine strains of Actinomyces

Of the 11 strains of Actinomyces isolated from cattle, one strain, A601, was identical in its characteristics with *Actinomyces israelii* as described by Howell *et al.* (1959). It served, therefore, as a comparative control and will be discussed later in regard to specific experiments. Ten strains, however, formed a homogeneous group which were essentially identical. These strains are referred to as *A. bovis*. Of the strains of *A. bovis*, only those isolated from cases P1, P2, P3 and P4 were studied from their initial isolation from infected cows (Pine & Watson, 1959). Since these strains encompassed the greatest range observed of variation in colonial morphology, it is considered important to describe these cultures completely. The conclusions based on their study were found to apply equally to the remaining strains.

Characteristics of clinical material from cases P1, P2, P3 and P4

The clinical specimens from which the P strains were isolated were cow heads exhibiting typical lumpy jaw. The upper jaws showed tumour-like enlargements of the bone which were invaded by multiple draining sinuses or which contained large abscesses filled with caseous material. When the pus from such sinuses was suspended in sterile distilled water and shaken, numerous 'sulphur' granules immediately sedimented. These, when mounted in 10% (w/v) KOH solution, had a hard gritty consistency when rubbed and crushed between the coverslip and glass slide. When they were observed under low power of the microscope, the actinomycotic granules had a typical dense outline as shown in Pl. 1, fig. 1, and contained numerous densely packed 'clubs' (Pl. 1, fig. 2). When stained by Gram's method, Gram-positive actinomycotic filaments were seen at the borders of the dense central portions of the actinomycotic granule (Pl. 1, fig. 3).

Morphological aspects of colonies on primary isolation and of laboratory strains of Actinomyces bovis

Previous reports of other workers (Wright, 1905; Emmons, 1935), and our own experience, had emphasized the importance of observing the highly diagnostic spider-like microcolonies of Actinomyces (e.g. Pl. 1, fig. 4). On the isolation plates of strain P2 such colonies were found, although these were extremely minute (Pl. 2, fig. 13). However, for cases P1, P3 and P4 no such colonies were found, although repeated isolations were made at several month intervals from frozen pus (Pine & Watson, 1959). In the descriptions which follow, it is emphasized that the distinction between R and S colonies was based on the microscopic appearance of 24–48 hr. colonies as viewed by transmitted and reflected light. Smooth colonies were typically those shown in Pl. 1, fig. 5, and Pl. 2, fig. 10, while the term rough colony was used to designate the range of forms depicted in Pl. 1, fig. 6, Pl. 2, fig. 13 (spider forms), and Pl. 4, fig. 28. The differentiation of smooth and rough forms by transmitted and reflected light is shown in Pl. 4, figs. 26 and 27.

All the colonies from case P1 were small, circular, and with entire edges. The surfaces of the colonies were smooth, slightly convex, and moist in appearance but when photographed were similar to those shown in Pl. 2, fig. 11. These smooth colonies of case P1 were, on primary isolation, similar in appearance with smooth colonies of P2, P3 and P4 isolated subsequently and with the colonies observed initially from the laboratory strains A1, A10 and A11. When these smooth colonies were observed by transmitted light, they showed characteristically either a very dense 'hyphal' formation in the centre (Pl. 1, fig. 5) or a slightly concave centre with a small button. Consequently, the latter colonies generally had the appearance of a small torus (Pl. 2, fig. 10) by transmitted and reflected light. In the case of some strains, they resembled flat or slightly convex circular disks having a small projection in the centre (Pl. 2, fig. 12). The very small smooth colonies of several strains were of such a nature that clear sharp photographic images could not be obtained by reflected light (Pl. 2, fig. 11). This was attributed to the very mucoid nature of the surface of the colony at this time, or to the possibility that the edges of the colony merged so gradually with the agar surface that no sharp line of demarcation was formed, As these colonies become larger, however, or in the case of smooth colonies of other strains, the surface and edge of the colonies became more distinct (Pl. 2, fig. 16). In texture, the small smooth colonies were extremely soft and were easily broken when touched with an inoculating needle, while the larger smooth colonies maintained their structure when pushed short distances with the needle.

In case P1, only one type of colony, the smooth colony, was observed on primary isolation. However, on the primary isolation plates of cases P3 and P4, a second type of colony, the rough colony, was observed. When small and viewed by transmitted light, this colony was much more dense and more irregular in its outline than the smooth colony, and presented a rough granular appearance (Pl. 1, fig. 6; Pl. 4, fig. 27). Similar colonies were also observed in the laboratory cultures A8, A9 and A12. As shown by oblique lighting, when rough colonies enlarged, they became opaque and considerably more elevated than the smooth colonies, although they continued to be circular in form (Pl. 1, fig. 7; Pl. 4, fig. 26). The surface of such colonies was generally smooth, but in many instances their surface became folded and irregular (Pl. 4, figs. 28, 30) or often resembled an inverted raspberry. When such a colony was picked for transfer, it was easily removed as a whole. These colonies were quite hard and maintained their colonial morphology through considerable manipulation. However, when these rough colonies were examined under oil immersion, contrary to the expectation of finding interwoven mycelial elements, only masses of diphtheroid organisms were observed (Pl. 1, fig. 9).

When the isolation plates of case P2 were studied, the typical young smooth colonies observed on the isolation plates of the other cases were found (Pl. 2, figs. 12, 16). However, in this case the rough colonies were replaced by spider forms (P2R; Pl. 2, fig. 13) which rapidly coiled (Pl. 2, fig. 14) to form smooth, round, opaque colonies like those of P2S (Pl. 2, figs. 16, 17). Upon isolation of the rough colony, it was found that it did not grow significantly upon a second transfer unless a medium was used which consisted of a mixture of brain heart infusion agar and maintenance medium. Even then its growth was extremely slow and limited and required 7–10 days to reach a maximum as compared to 18–24 hr. required by the smooth colonies. Brain heart infusion agar did not support growth for more than two transfers. From Pl. 2, fig. 17, it is seen that the rough colonies of strain P2 are circular and raised, differing somewhat from the rough colonies of P3 and P4 in the surface texture and in being more convex (Pl. 4, fig. 28).

Repeated isolations were made from cases P1, P2, P3 and P4 to verify the presence or absence of spider-like colonies. Of these only P2 exhibited spider colonies. Representative colonies of each type from the various strains were picked and cultured by the methods described earlier in this paper. From case P1, only one stock culture was made, i.e. *Actinomyces bovis* P1S; from case P2 a smooth colony subculture, *A. bovis*, P2S, and a rough (spider) colony subculture, *A. bovis*, P2R; from case P3, a smooth colony subculture, *A. bovis* P3S, and a rough colony subculture, *A. bovis* P3R, were made; while from case P4, only a single culture, *A. bovis* P4R, was made, even though smooth colonies were observed.

Cultural characteristics of Actinomyces bovis *in liquid media*

Several experiments with liquid media were done to determine the effect of the medium and conditions of growth on the cultural characteristics of the strains of *Actinomyces bovis.* In these experiments the Casitone containing medium, the defined medium + casein hydrolysate, and the maintenance medium were used with aerobic or anaerobic carbon dioxide seals (Pine & Howell, 1956). Organisms in such tubes of liquid medium settled to the bottom; the growth was either mucoid, i.e. forming spiralling viscous ropes when the contents of the tubes were swirled, granular, i.e. forming bread-crumb colonies similar to those described for *A. israelii*, or flaky, i.e. resembling flakes of snow when the tubes were shaken. In continuous transfer, the types of growth of a single strain in any one medium could vary from mucoid to flaky, to granular. Such changes were observed to occur in all of the media, although growth in the casein hydrolysate medium was slow (maximum growth in 3–5 days) and tended to become granular in the presence of air and carbon dioxide. On the other hand, anaerobically, growth in the maintenance medium was generally mucoid or flaky and reached its maximum within 2–3 days.

Cellular morphology of Actinomyces bovis

With the exception of isolate P2R the cellular morphology of the *Actinomyces bovis* strains was uniformly diphtheroid in liquid media or on agar media (Pl. 3, fig. 20), and, in general, distinguishable from the usual rods observed for most strains of *A. israelii* (Pl. 3, fig. 18) or *A. naeslundii*, as recently shown by Howell *et al.* (1959). The diphtheroid forms of *A. bovis*, however, did resemble the diphtheroid forms of *A. israelii* (Pl. 3, fig. 19) isolated from lacrimal infections (Pine & Hardin, 1959).

From the usual agar media, it was difficult to ascertain the true mycelial nature of the bovine isolates in their initial stages of growth, although branching diphtheroid cells were invariably present. To demonstrate the different morphological types and, in particular, the mycelial phases of growth, the strains were grown in liquid maintenance medium containing 0·05 % (w/v) agar. The colonies were then drawn into capillary pipettes and transferred to glass slides, heat fixed and Gram stained or mounted in lactophenol cotton-blue mounting medium (Conant *et al.*

1954). When mounted in lactophenol, the colonies characteristically showed compact masses of growth from which one or two filaments extended (Pl. 3, fig. 24), but which did not demonstrate the true mycelium as first shown by Wright (1905) for human isolates under similar conditions. When the culture was of a rough strain, these compact masses retained their structure, whereas smooth strains tended to disintegrate into individual diphtheroid elements.

When stained by Gram's method, very young single colonies of the rough forms often showed a definite mycelium (Pl. 3, fig. 25), which rapidly became unapparent with further growth. Gram stains of the smooth strains grown in maintenance liquid medium + 0·05 % (w/v) agar often showed an indefinite cellular structure (Pl. 3, fig. 22), and the presence of many bizarre forms which were similar if not identical with those described by Sundman & Björksten (1958) for *Lactobacillus bifidus*. As the diphtheroid forms of the rough and smooth strains entered the maximum stationary phase of growth, they became partially Gram negative and often exhibited multiple round metachromatic granules distributed within the cells (Pl. 3, fig. 21). As shown in Pl. 3, fig. 23, the morphology of the mycelial *Actinomyces bovis*, strain P2R, was similar to that found with the typical *A. israelii* forms (Pl. 3, fig. 18) but the bacillary elements were finer.

Factors influencing colonial morphology of strains of Actinomyces bovis

Within several months after their initial isolation and purification it became evident that various strains of *Actinomyces bovis* were changing in their colonial morphology. Rough cultures exhibited a mixture of smooth and rough colonies and occasionally smooth cultures would show rough colonies. By direct experiment, or indirectly from general observations made throughout this work, several factors were observed to effect the kind of colony which might result from a purified strain. These factors were the strain used, medium used, number of transfers undergone by the strain, and the effect of animal inoculation. These results are presumably an expression of selection due to nutritional factors, genetic variation, or both.

Colony morphology as related to strain characteristics. Because of the extreme morphological diversity of strains P2S and P2R, it was thought to be desirable to establish, if possible, the origin of the two types. Either a mixed infection with *Actinomyces israelii* and *A. bovis* had occurred in the cow or one form was a variant of the other. Consequently, the purity and the stability of the two forms were established. After three consecutive transfers consisting of streaking and re-isolation of single microcolonies, a strain of the P2 rough spidery colony type (P2Rb) was obtained which did not form smooth colonies. No change in the morphology or character of this culture has been observed in a period of 2 years. In the case of the smooth form, it was found that single colonies of P2S formed only smooth colonies when restreaked and a stock culture P2S was made from these. Likewise, no changes in the colony characteristics of this strain were observed during 2 years. However, recently rough colonies similar to those seen in Pl. 1, fig. 6, have been seen in P2S.

With the exception of strains P2S and P2R, most of the strains of *Actinomyces bovis* showed a ready conversion of rough to smooth or in a few strains a much more limited conversion of smooth to rough. In attempts to induce such a conversion in the P2R cultures, strain P2R and several single colony isolates from it were grown in maintenance liquid medium + brain heart infusion, or in thioglycollate broth. When growth was heavy, a loopful of inoculum was streaked on maintenance or brain heart infusion agar plates. The colonies which appeared were identical with the stock cultures from which they originated. Rapid passage for six consecutive transfers in thioglycollate broth and in the mixture of maintenance liquid medium + brain heart infusion did not induce any permanent change in colony morphology, even though cellular morphology was observed to vary from purely mycelial strands to diphtheroid cells. Similarly, rapid transfer of P2S in maintenance broth produced cells which when streaked on maintenance agar plates formed rather granular smooth colonies. These were, nevertheless, quite different from the spidery rough colonies with which they had been associated in the cow.

In addition to the above experiment, each of 10 plates were inoculated with 0·2 ml. of a maintenance liquid culture of P2S and 10 plates with 0·2 ml. of similar rough P2R cultures. Critical examination of all plates still failed to demonstrate any colonies which indicated a mutation of P2R to P2S or vice versa.

Stock cultures of the other strains (P1S, P3R, P3S, P4R), which had been purified by picking a single microcolony for further propagation, did not retain their colonial homogeneity. For example, after being transferred at monthly intervals during one year, the stock culture of P3R was streaked on maintenance medium agar. As shown (Pl. 4, fig. 28) the colonies which developed were a mixture of smooth and rough colonies, whereas a similar streaked plate of P3S had smooth colonies only (Pl. 4, fig. 29). From a streak plate of P3R, 6 smooth colonies and 6 rough colonies were picked. Each was grown in maintenance liquid medium and each culture was streaked on agar plates. On the resulting plates, only smooth colonies were found from the P3S culture. On the plates inoculated with the rough cultures, from 1 to 15 smooth colonies were found. A rough colony was picked from such a plate, and a stock culture (P3Rbc) was made. Subsequently, maintenance of this culture resulted in a gradual increase in the ratio of smooth:rough colonies, whereas continued transfer of P3Sb (made from a smooth colony of the P3S plate) showed a small but definite gain in the rough variant. Of the strains studied, the change was generally rough to smooth, although in the case of strain A11, the reverse was true. Strain P1S, which was originally isolated as a pure smooth colony, was found to be predominantly a rough culture after 2 years maintenance.

As shown in Table 2, the direction of the change in colonial morphology depended upon the strain tested. In this experiment stock cultures of the rough and smooth strains, all of which originated from single microcolonies, were restreaked on two plates of brain heart infusion agar and two plates of maintenance medium agar. The ratio of smooth colonies to rough colonies, or vice versa, in eight separate fields of each plate was determined. It is concluded that changes in the P3 strains are best expressed diagrammatically as P3R $\overset{\leftarrow}{\longrightarrow}$ P3S and in the A11 strains as A11S $\overset{\leftarrow}{\longrightarrow}$ A11R. Again, in the case of the P2 strains, a change of colonial morphology in either direction was not observed.

Effect of medium on colonial morphology. As shown in Table 2, the medium used may affect the relative number of microscopic smooth or rough colonies. It was observed during the primary isolation of the bovine strains that colonies on brain heart infusion agar were uniformly smooth although very small. Isolations made on maintenance medium agar, however, showed some rough colonies.

Different media may also affect the gross colonial morphology of a given strain. For example, a direct comparison of the effect of several media on colonial morphology was made with rough and smooth strains of *Actinomyces bovis.* As shown in Pl. 4, fig. 30, large colonies of P3S on thioglycollate agar had a highly irregular surface, whereas on maintenance medium agar the colony was circular with slight furrowing of the smooth surface (Pl. 4, fig. 31). Strain A11 often formed only smooth colonies on brain infusion agar, whereas the same inoculum streaked on maintenance medium agar formed smooth and rough colonies. It is emphasized that the large rough colonies of *A. bovis* may often be identical to those of *A. israelii* and *A. naeslundii* as depicted by Howell *et al.* (1959).

Effect of animal inoculation on colonial morphology. Sixteen hamsters were inoculated with strain P2S in several different experiments; isolation plates made from the pus of one animal in an early experiment showed many P2R colony types. However, pus from two other animals of later experiments showed only four rough colonies in a total of 24 streaked plates which were heavily covered with colonies of P2S. In several experiments in which hamsters were inoculated with P2R, only the original type of spidery colony was obtained.

Table 2. *Relative proportion of rough and smooth colonies of* Actinomyces bovis *found on streaked plates* 6 *months after single colony isolation*

Two plates of each medium brain heart infusion agar (Difco); maintenance agar (Pine & Watson, 1959) were made for each strain. Inoculum was taken from stock cultures with a loop, suspended in a few drops of water, and streaked on the agar plates. Plates were incubated 5 days in a 5 % $CO_2 + 95$ % N_2 atmosphere at 37°. Plates were examined with a microscope using scanning objective (×5) and wide field oculars (×15). Eight different fields were counted per plate.

	Ratio of S:R on	
Strain	BHI	Maintenance
P3S	27:1	27:1
P3R	7:3	6:7
A11S	1:10	1:5
A11R	1:37	1:40
P2S	500:0	500:0
P2R	0:500	0:500

Other strains of *Actinomyces bovis* also showed increase of smooth to rough colonies in the animal. In one experiment (Table 3), cultures of various strains were grown in maintenance medium broth; two loopfuls of medium were then streaked on duplicate plates of brain heart infusion agar and of maintenance medium agar. The remainder of each culture was used to inoculate one hamster per strain. Pus was taken from the skin abscesses formed initially or from internal abscesses observed at autopsy 1 month after inoculation. The pus was streaked on duplicate plates of brain heart infusion agar and maintenance medium agar and Gram stains were made to examine for mycelium in the pus. As seen from Table 3 the initial liquid cultures of strains P1S, P3R, A10, A11, and A12 consisted of smooth, or mixed smooth and rough populations. However, upon the isolation from hamsters, the colonies obtained were predominantly rough. On the other hand, strains P2S and P3S did not show this propensity for smooth to rough increase.

Relative pathogenicity of Actinomyces bovis *and human strains of Actinomyces*

In Pl. 4, figs. 32 and 33, it is seen that *Actinomyces bovis* strains formed typical actinomycotic branching mycelial elements when inoculated into animals. However, as indicated in Table 3, the ability of these strains to form mycelium in the animal is apparently much more limited than that of the human strains of Actinomyces.

Table 3. *Variation of colony morphology of* Actinomyces *spp. as a result of hamster inoculation*

Growth of inoculum was observed on agar plates within 24–48 hr. by transmitted light (see text). S–smooth *Actinomyces bovis* type of colony, R–rough *A. bovis* type of colony, N–*A. naeslundii* type of colony, I–*A. israelii* type of colony. Relative amount of mycelial elements formed was observed by Gram stains of pus removed from skin lesions 7–15 days after inoculation. Pathology was observed at autopsy 21 days to 1 month after inoculation. Colony type on isolation on maintenance agar was observed on plates streaked with pus from skin lesions taken 7–15 days after inoculation.

	Colony type on		Relative amount of mycelial elements formed	Pathology at autopsy	Colony type on isolation on maintenance agar
Strain	BHI	Maintenance			
P1S	S	S	+1	2 large abscesses in skin	S+R
P2R	I	I	+1	1 large abscess in skin	I
P2S	S	S	0	1 small abscess in skin	S
P3S	S	S	0	None	S
P3R	S+R	S+R	0	None	R+few S
P4R	S+few R	S	+2	None	S+few R
A10	S+few R	S+R	0	None	R+few S
A11	S+R	S+R	+1	1 small abscess in peritoneal wall	R
A12	S+R	S+R	+1	Several small abscesses in peritoneal wall	R
279	N	N+S	+5	Multiple abscesses in mesenteries	N
286	S	N	+1	None	N
296	N	I	+5	None	Not done
295	I	I	+5	Several small abscesses in body wall	I
261	Not done	Not done	.	Single small abscess in mesenteries	Not done
262	N	N	+5	Few small abscesses in mesenteries	I
263	N	N	+5	Single small abscess in body wall	I
282	Not done	Not done	+5	Few abscesses in mesenteries; massive abscesses in peritoneum	I
283	I	I	+5	Single abscess in body wall	I
287	—	I	0	Single abscess in body wall	Not done
534	I	I	+3	1 large caseous abscess in body wall, sulphur granules	I
895	I	I	+5	Few abscesses in mesenteries; few abscesses in peritoneal wall	I
699	I	I	+5	Several large abscesses in internal organs	I
OS131	I	I	+5	Several large abscesses in internal organs	

It was generally difficult to find Gram-positive filaments in an animal inoculated with *A. bovis* unless an abscess formed which remained for 2 weeks or more (Table 3). All bovine strains tested, however, have at one time or another formed lesions containing typical mycelial elements when inoculated into animals. On the other hand, one can invariably observe long mycelial branching forms of *A. israelii* or *A. naeslundii* strains in young lesions, even though the lesion is short-lived and heals spontaneously. As shown by Table 3, many of the human strains of Actinomyces produced abscesses which were present 21 days to 1 month after inoculation, while only a few of the bovine strains did so.

Physiological and biochemical characteristics of bovine strains of Actinomyces

Characteristics of growth. Excepting strains A601 and P2R, all strains had a relatively high rate of growth, reaching maximum amount of growth within 30–36 hr. in Casitone containing liquid medium. This rate of growth is not as rapid as that exhibited by some strains of Actinomyces (Howell & Pine, 1956), which were subsequently identified as *Actinomyces naeslundii* (Howell *et al.* 1959). It was in general much more rapid than the growth exhibited by the typical *A. israelii* strains.

Table 4. *Effect of size of inoculum on growth of bovine strains of Actinomyces*

Maximum growth expressed as optical density obtained in 7 days with continuous shaking. Carbon dioxide was supplied as a $Na_2CO_3 + KH_2PO_4$ or Na_2CO_3 + pyrogallol seal. 0·05 ml. of inoculum was used per tube containing 5 ml. of casitone broth.

	Inoculum O.D. = 0·5			Inoculum O.D. = 0·0005	
Strain	Aerobic	Aerobic $+CO_2$	Anaerobic $+CO_2$	Aerobic $+CO_2$	Anaerobic $+CO_2$
P1	0·03	1·58	1·65	0·03	1·49
P2S	0·01	0·96	1·85	0·03	1·82
P3R	0·03	0·63	0·65	0·07	1·85
P3S	0·01	1·63	1·82	0·01	1·88
P4	0·07	1·30	1·66	0·07	1·60
A1	0·04	0·53	1·21	0·02	1·39
A8	0·07	2·00	1·25	0·01	1·11
A9	0·02	1·10	2·00	0·00	2·00
A10	0·01	0·02	1·30	0·01	1·33
A12	0·02	0·00	0·92	0·02	1·47
A601	0·01	2·00	2·00	0·00	2·00

As shown in Table 4, large inocula of most strains grew in air when the concentration of carbon dioxide was increased. However, small inocula required anaerobic conditions for growth. Neither the rates of growth nor yields of organisms formed by strains of *Actinomyces bovis* (except strain A8) were greater in the presence of air + carbon dioxide than anaerobically. This result distinguishes *A. bovis* from strains now recognized as *A. naeslundii*, which may show an increased amount of growth when carbon dioxide + air are present (Pine & Howell, 1956).

General physiological characteristics. Excepting strain A601, all strains, whether smooth or rough variants, exhibited the same general physiological characteristics. They were catalase negative, did not liquefy gelatin in the presence or absence of glucose, did not reduce nitrate to nitrite and did not form hydrogen sulphide or indole. The production of acetoin was variable. Litmus milk was often reduced, but little if any acid curd was formed. The casein was never coagulated or peptonized. None of the strains grew in 4% (w/v) sodium chloride or in 20% (w/v) bile.

Perhaps the most striking physiological character observed in the *Actinomyces bovis* strains as compared with the human strains was their ability rapidly to hydrolyse and ferment starch and to use it as a carbon source for growth. The amount of starch hydrolysis in 24–72 hr. by human strains was negligible or limited to immediate vicinity of the colonies; the bovine strains gave total hydrolysis of streak plates or slants within 24–48 hr. When agar plates of maintenance medium with 0·5% (w/v) starch and no added glucose were streaked with Actinomyces strains, all of the strains of *A. bovis* grew in 3 days and when tested with KI + I solution showed complete hydrolysis of the starch. On the other hand, strain A 601 behaved similarly to the human strains. The human strains either failed to grow, grew poorly with no hydrolysis of the starch, or grew slowly with very limited zones of hydrolysis surrounding heavy areas of growth. In Casitone broth in which 0·5% starch replaced the glucose, all the strains of *A. bovis* hydrolysed starch and formed acid (Table 5). However, none of the human strains of Actinomyces turned the medium acid, although cultures OS 131, 286, 279 and 283 effected some hydrolysis of starch as indicated by the formation of a deep red iodine-starch complex. Strain A 601 did not grow on this medium.

The strains of *Actinomyces bovis* formed a homogeneous group which gave acid but no gas with glucose and starch (Table 5). They formed acid from fructose, maltose, lactose, and sucrose less consistently, half of the strains fermented mannose and an occasional strain attacked glycerol. None of the other carbohydrates tested gave acid or gas. In contrast, strain A 601 gave strong acid formation with fructose, maltose, lactose, sucrose, mannose, xylose, ribose, raffinose and salicin. Rhamnose gave some acid; there was no acid from starch. This result with strain A 601 is similar to the results obtained previously with the various human strains (Pine & Howell, 1956; Howell *et al.* 1959). These results, its microcolony morphology, and other characteristics justified the conclusion that strain A 601 was a strain of *A. israelii.*

Twenty to 29 μmole glucose were fermented by 9 strains of *Actinomyces bovis* with the formation of formic acid (6–9 μmole); acetic acid (6–10 μmole), succinic acid (5–12 μmole) and lactic acid (31–49 μmole); carbon recoveries ranged from 86 to 103%. Isolation and determination of the specific rotation of the lactic acid of *A. bovis*, strain A 12, showed that the acid was L(+) lactic acid. These are the same products as reported previously for the human strains of Actinomyces (Pine & Howell, 1956). As shown previously for human strains, there was an uptake of carbon dioxide during growth of *A. bovis*.

DISCUSSION

Cohn (1875) first observed and described a branching filamentous micro-organism in the concretions taken from a lacrimal canal. This organism, now recognized as *Actinomyces israelii* (Smith, 1953; Pine & Hardin, 1959) was named *Streptothrix foersterii*. This statement raises a question of taxonomic priority. However, the genus name *Streptothrix* is invalid since it was originally used for a different genus of fungi by Corda in 1839 (Breed & Conn, 1919). The name *Streptothrix* (Cohn, 1875) is listed as a synonym for *Streptomyces* in *Bergey's Manual*, 6th and 7th ed. To the best knowledge of the senior author, no species of *Norcardia* or *Streptomyces* has been identified as a cause of lacrimal canaliculitis, although these organisms have been responsible for other infections of the eye. Since it has been established that *A. israelii* is the primary cause of concretions in the lacrimal canal with subsequent lacrimal canaliculitis and blepharitis (Smith, 1953; Pine, Hardin, Roberts & Turner, 1960) it is our opinion the species name *foersterii* has priority over *israelii*. However, we feel that common usage warrants the continuation of the present nomenclature.

Table 5. *Result of fermentation of specified sugars by bovine strains of Actinomyces*

+ = Acid, no gas, indicator (bromcresol purple) yellow, final pH 5·0–5·5. ± = indicator violet, moderate acid formation, final pH 5·5 to 6·0. − = indicator purple (unchanged), little or no acid formation. Test tubes incubated 7 days at 37°. Duplicate values given are comparative results of two different laboratories. Basal medium was the casitone medium with 0·5 % (w/v) sugar.

	Substrate							
Strain	Glucose	Starch	Fructose	Maltose	Lactose	Sucrose	Mannose	Mannitol
P1	+, +	+	+	+, +	+, +	+, +	+	−, −
P2R	+	+	+	+	+	+	−	−
P2S	+, +	+	+	+, +	+, +	+, +	+	−, −
P3R	+, +	+	+	+, +	+, +	+, +	±	−, −
P3S	+, +	+	+	±, ±	+, +	+, +	−	−, −
P4	+, +	+	±	+, +	+, −	+, ±	−	−, −
A1	+, +	+	+	+, +	+, ±	+, +	−	−, −
A8	+, +	+	−	−, ±	+, ±	+, +	−	−, −
A9	+, +	+	+	±, ±	+, +	−, +	±	−, −
A10	+, +	+	+	±, ±	+, ±	−, +	+	−, −
A11	+, +	+	+	±, −	+, +	+, +	−	−, −
A12	+, +	+	+	+, −	+, ±	−, +	+	−, −
A601	+, +	−	+	+, +	+, +	+, +	+	−, −

	Substrate							
Strain	Xylose	Arabinose	Ribose	Raffinose	Inulin	Salicin	Glycerol	Rhamnose
P1	−, −	−	−	−	−	−, −	−	−
P2R	−	−	−	−	−	−	−	−
P2S	−, −	−	−	−	−	−, −	−	−
P3R	−, −	−	−	−	−	−, −	−	−
P3S	−, −	−	−	−	−	−, −	−	−
P4	−, −	−	−	−	−	−, −	−	−
A1	−, −	−	−	−	−	−, −	−	−
A8	−, −	−	−	−	−	−, −	−	−
A9	−, −	−	−	−	−	−, −	−	−
A10	−, −	−	−	−	−	−, −	−	−
A11	−, −	−	−	−	−	−, −	±	−
A12	−, −	−	−	−	−	−, −	−	−
A601	+, −	−	+	+	−	+, +	−	±

Shortly after the description made by Cohn, Harz (1879) described in detail a similar organism in granules observed in tissues of cows with lumpy jaw. Previously, he had named this organism *Actinomyces bovis* (Harz, 1877). Neither Cohn nor Harz cultured the organisms observed by them, and it was not until approximately 10 years later that the organism was isolated and cultured anaerobically from lumpy jaw of cow by Mosselman & Liénaux (1890) and from actinomycosis of man by

Wolff & Israel (1891). Because of the pathological relationships of the two diseases as shown by Israel (1878) and Ponfick (1880) and the similarity in the formation of granules in tissue, it was considered that the organisms causing these diseases were the same. This opinion was later supported by Silberschmidt (1901) who compared the cultural and morphological characteristics of pure cultures taken from a case of lacrimal infection of man, thoracic and maxillary actinomycosis in man, and lumpy jaw of a cow; and by Wright (1905) who compared two cultures isolated from two cases of lumpy jaw of cows with 13 cultures taken from actinomycosis in man. Both investigators considered the strains isolated from man and cow to be identical. Consequently, after the taxonomic validity of the name had been established by Breed & Conn (1919), the name *A. bovis* was applied to all strains of anaerobic actinomycetes isolated from actinomycosis of man or animals.

Erikson (1940) compared 5 strains of *Actinomyces bovis*, isolated from infected cows, with 15 strains isolated from human sources. She found that the bovine strains formed a homogeneous group which, without exception, produced only minute, flat, smooth, soft colonies on adequate media. Microscopic examination of the margins of these colonies did not reveal the irregular radiating outgrowths characteristic of the human strains as a whole; instead, they showed an almost entire margin. When extrusion of filaments did occur, it took place in a sinuous arrangement and not in the usual radiating pattern. On the basis of these results and the facts that growth in liquid was more turbid, that the animal strains were more oxygen tolerant, and that these strains had a lesser ability to ferment salicin and mannitol, she divided the genus into two species. *A. bovis* was the name applied to the smooth animal strains; whereas, *A. israelii* Kruse was used for the strains isolated from man. The association of the smooth *A. bovis* with pulmonary actinomycosis in swine was established by Vawter (1946), who also pointed out the absence of myceloid or filamentous colonies. Subsequently, Thompson (1950) isolated 11 strains of *A. bovis* from cattle with lumpy jaw and compared them, in very limited experiments, with 8 strains isolated from human infections. All of the strains isolated from cows formed smooth round colonies after 6–7 days except one which was identical with the rough strains of man. However, contrary to the results of Erikson (1940), Thompson (1950) observed that the very young colonies were composed of mycelial elements which radiated from the centre of the colony. The strains of *A. bovis* grew diffusely in thioglycollate medium, although in brain broth growth was slow and limited to the bottom of the tube. On the basis of his studies, Thompson (1950) concluded that Erikson's separation of Actinomyces into the two species was justified.

Later, Thompson & Lovestedt (1951) described strains of Actinomyces which grew in air on primary isolation. Subsequently these strains formed rough and smooth colonies and grew rapidly and profusely on suitable media. For this group, they proposed the name *Actinomyces naeslundi*. Recently, Howell *et al.* (1959) have more clearly defined and delineated this group, for which they also support the name *A. naeslundii*.

However, the results of many investigators emphasized that colonial and cultural descriptions of many strains of *Actinomyces* spp. were subject to variation with changes in medium or conditions of growth (Rosebury, 1944). In addition studies by Slack, Spears, Snodgrass & Kuchler (1955) indicated that Actinomyces isolated from horse, cow, pig, and man could not be differentiated immunologically. Some investigators, therefore, have not considered the difference between *Actinomyces bovis* and *A. israelii* to be sufficiently great to warrant species separation and continued to use the single name *A. bovis* for all strains, even though such species separation was given in *Bergey's Manual*, 6th (1948) and 7th (1957) editions. Indeed, this was also our opinion at the initiation of this work.

However, when we first observed the entire dew-drop colonies of *Actinomyces bovis* on isolation plates they were strikingly different from the small mycelial colonies which we anticipated from human cases of actinomycosis. Consequently, there was no complete assurance that these were, indeed, strains of Actinomyces until each culture was later observed to form typical actinomycotic mycelium when inoculated into hamsters or mice. The original colonies were chosen because they represented the majority of the colonies observed or were the only organisms isolated, were catalase negative, and were made up of Gram-positive diphtheroid elements. These forms were then recognized as being related to forms of *A. israelii* and *A. naeslundii*, but additional studies emphasized differences in animal pathogenicity, cellular morphology and fermentation of carbohydrates. In addition, a high rate of change from smooth to rough or rough to smooth was common to most of the strains of *A. bovis*, but not observed with strains of *A. israelii*.

Erikson (1940) also reported that human strains of Actinomyces failed to form smooth colony variants. However, Lentze (1938) isolated rough and smooth strains from human cases of actinomycosis and by direct observation of single microcolonies observed the conversion of rough colonies to smooth colonies. The colonies described by Lentze as type II*a* were morphologically identical to the smooth colonies of our *Actinomyces bovis* strains. However, the colony forms of the typical Wolff-Israel Actinomyces described and photographed by Lentze were essentially identical to the *A. israelii* strains which we have depicted. Rosebury, Epps & Clark (1944) have also described what may have been a rough to smooth variation.

Vawter (1946) described the colonies formed by strains isolated from pulmonary actinomycosis of swine as being opalescent, pulvinate, homogeneous forms with smooth edges. Myceloid or filamentous colonies were not observed on agar plate cultures. The colonies photographed by Vawter (1946) were identical with those in our Fig. 12 (Pl. 2); he did not report any colony variation.

Thompson (1950) did not observe smooth to rough conversion in any of the bovine strains he isolated, although Thompson & Lovestedt (1951) have described rough to smooth and smooth to rough change in *Actinomyces naeslundii*. Such colonial variation was not described by Magnusson (1928) for his type A, isolated from cows. However, Magnusson (1928) reported two types of cultures isolated from infected sow udders. One, type B, was typical of the cultures described by Wright (1905) for *A. bovis*, being hard, granular, and strongly adherent to the surface of the agar medium. The second type (type C) was soft, succulent, and irregularly round, and the colonies were 5 to 10 times larger than those of type B. Types B and C were found together in the same abscess in 11 cases, while B was found alone in 99 cases, and C was found alone in 71 cases. Unfortunately, cultural and colony descriptions of these strains were limited; no photographs of the colonies were presented, and, due to the inability to grow the isolates, few physiological data were obtained. Consequently, the colonial and physiological relationships of types A, isolated from cows, and B and C, isolated from swine to the smooth and rough forms of the bovine strains described here cannot be ascertained.

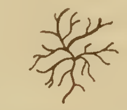
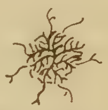
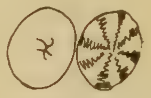
Although colony changes of any given strain may be readily observed, the factors and the interrelationships responsible for such changes are not, by any means, completely understood. When such changes occurred, the rough microcolonies of *Actinomyces bovis* still did not show a true radiating mycelium as did *A. israelii* colonies. On the other hand, *A. naeslundii*, which generally forms colonies having true mycelium, will sometimes form small microcolonies indistinguishable from those of *A. bovis* (Howell *et al.* 1959). However, other characters readily separate this proposed species from *A. bovis*. Further identification by means of colony morphology may be made by the use of brain heart infusion agar. The ability of brain heart infusion agar to support the appearance of smooth forms of *A. bovis* has been so consistent on primary isolation from animals or primary transfer from the maintenance medium that it serves as a valuable diagnostic aid for separating *A. bovis* from *A. israelii*, even though brain heart infusion agar itself fails to maintain cultures beyond a few transfers (Pine & Watson, 1959).

The results of sugar fermentation studies reported by Ludwig & Sullivan (1952), King & Meyer (1957) and Hazen & Little (1958) for bovine isolates are not in agreement with our findings. However, one of the two bovine isolates described by King & Meyer, A 601, has been identified by us as *Actinomyces israelii*. Neither Thompson (1950) nor Magnusson (1928) gave data on sugar fermentations. On the other hand, our data are in agreement with those of Erikson (1940) for bovine strains but not with the very limited data of Menges, Larsh & Habermann (1953) in a case of canine actinomycosis in that their strain failed to ferment lactose. The fermentative results of Vawter (1946) with a strain isolated from swine differ from the results we obtained with bovine strains since the swine strain did not ferment starch. The bovine strains tested by him fermented sugars which were not fermented by our strains (Vawter, 1933). In addition, it reduced nitrate to nitrite. Kimball, Twiehaus & Frank (1954) ultimately showed that the Actinomyces strains reported by them to cause equine fistulous withers were catalase positive and therefore the sugar fermentative data (Kimball & Frank, 1945) cannot be considered as applicable to Actinomyces (Suter, 56). The remaining physiological data reported herein for *Actinomyces* spp. substantiate the homogeneity of the genus, there being little to separate the species *A. bovis*, *A. israelii* and *A. naeslundii*. However, no strain of *A. bovis* reduced nitrate to nitrite; all of the strains of *A. naeslundii* used here or described previously (Howell *et al.* 1959) did reduce nitrate to nitrite, while this characteristic was variable in strains of *A. israelii*.

Cummins & Harris (1958) reported the results of cell-wall studies of members of the Actinomycetales and related groups. In this study it was found that strains of Actinomyces typical of *Actinomyces israelii* contained galactose, glucosamine, muramic acid, and the amino acids alanine, glutamic acid and lysine. Strains isolated from cows, however, contained rhamnose, sugar component 'P', glucosamine, muramic acid and aspartic acid in addition to the amino acids alanine, glutamic acid and lysine. In some strains small amounts of galactose were also present. On request our bovine strains were sent to Dr Cummins, who determined the cell-wall composition and compared the results with those reported previously (Cummins & Harris, 1959). Again, it was found that on the basis of cell-wall studies, these cultures of *A. bovis* were essentially identical to the bovine strains studied originally. However, strain A601 gave an analysis typical of *A. israelii* strains.

In addition, Dr Cummins found no major difference between the rough and smooth variants (P2R, P2S and P3R, P3S) with the exception that P2S contained galactosamine and P2R had little if any aspartic acid (Cummins, 1959, personal communication). The results of Cummins & Harris (1959) not only re-emphasize the validity of the separation of these groups as proposed by Erikson (1940), but also support our conclusion, based on physiological data, that lumpy jaw in our bovine case P2 was not the result of a mixed infection with *Actinomyces israelii* and *A. bovis*. In view of the fact that reproducible conversion of P2S to P2R, or vice versa, was not obtained, the question as to the origin of these two colonial types in the P2 case of lumpy jaw remains unresolved. If the rough form occurred as a variant of the smooth colony, the rough form would reflect not only a loss of galactosamine, and a partial loss in aspartic acid in the cell wall, but also a requirement for growth factors, some of which are present in brain heart infusion but are not in the Casitone medium.

Table 6. *Characters identifying* Actinomyces *species*

Character	*A. israelii*	*A. naeslundii*	*A. bovis*
Morphology of microcolony (24–48 hr.)			
Aerobiosis	Anaerobic to micro-aerophilic	Facultative in presence of increased CO_2	Anaerobic to micro-aerophilic
Maximum growth* reached in	3–7 days	1 day	2–3 days
Catalase	–	–	–
Gelatin liquefaction	–	–	–
Starch hydrolysis	+, –	+, –	+10
Acid but no gas formed:†			
Glucose	+	+	+
Xylose	+(–)	–	–
Mannitol	+(–)	–	–
Raffinose	–, +	+	–
Starch	–(+)	–(+)	+
Nitrate reduced to nitrite	+(–)	+	–

* As tested in maintenance or Casitone broth.

† Values given in parentheses indicate this reaction in 12–20 % of strains tested.

Based on the results of Silberschmidt (1901), Wright (1905), Thompson (1950), and the results presented here with strain A601, there can be little doubt that *Actinomyces israelii* may be a cause of lumpy jaw in cattle. However, based on our results and those cases adequately described in the literature, the number of cases due to this species is small in comparison with the bovine infections caused by *A. bovis*. On the other hand, with the exception of the work reported by Lentze (1938) and Holm (1948), there is little evidence suggesting that *A. bovis* can cause actinomycosis in man.

In the many animal experiments done with mice and hamsters, the impression is obtained that the human strains of Actinomyces are more pathogenic than the bovine strains. This impression is a result of general observation of the mycelium formed in tissues and of the relative numbers of progressive infections resulting from animal inoculations. But as shown by Table 3, only 5 human strains formed an extensive infection. Therefore, any conclusion as to relative pathogenicity of the bovine and human isolates is limited by the erratic nature and by the very low percentage of infections caused by any member of the genus (Miller & Drake, 1951; Rosebury, 1944).

On the basis of the results reported here and previously (Pine & Howell, 1956; Howell *et al.* 1959) and by others (Erikson, 1940; Thompson, 1950; Thompson & Lovestedt, 1951), three main groups of *Actinomyces* can be differentiated from each other by the characters listed in Table 6.

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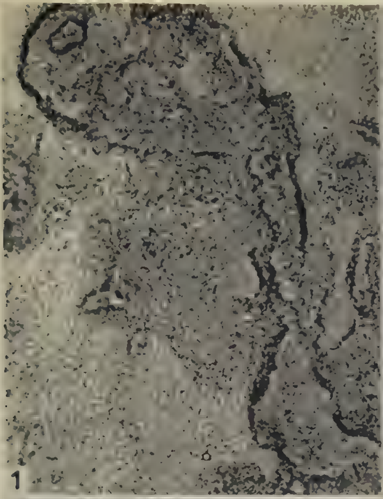
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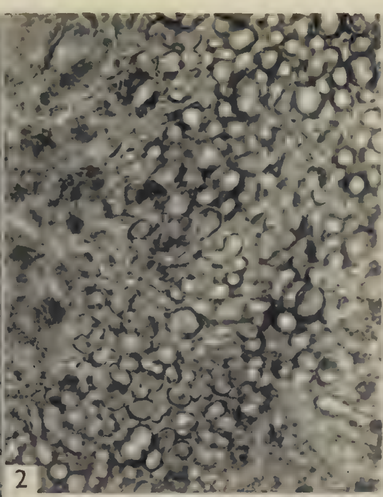
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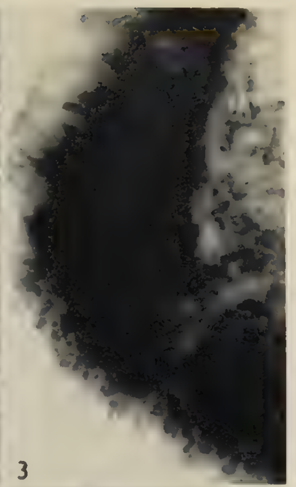
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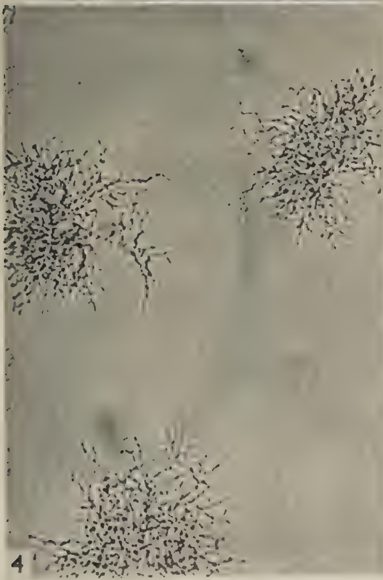
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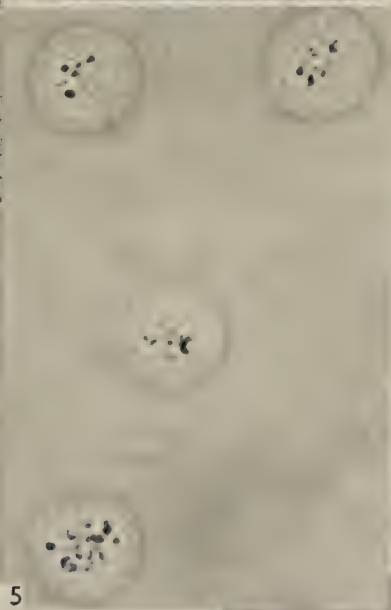
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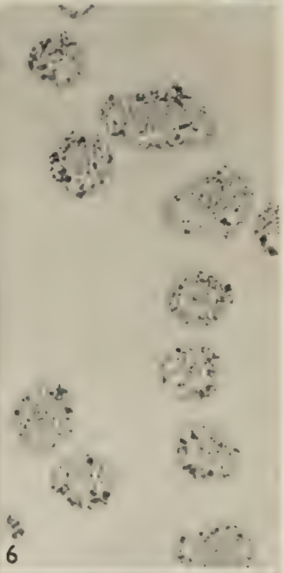
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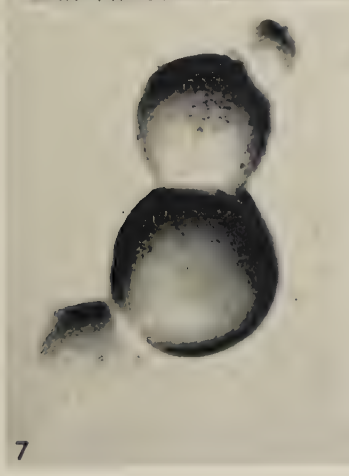
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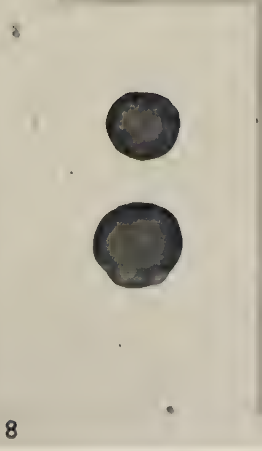
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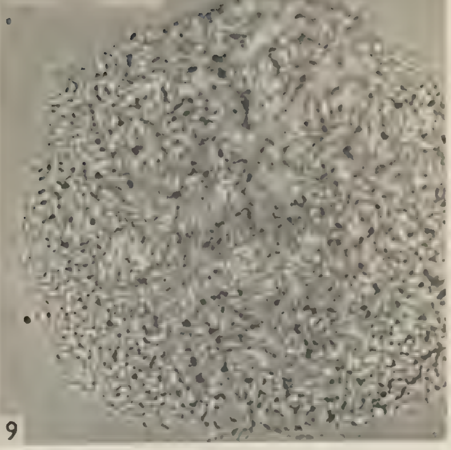
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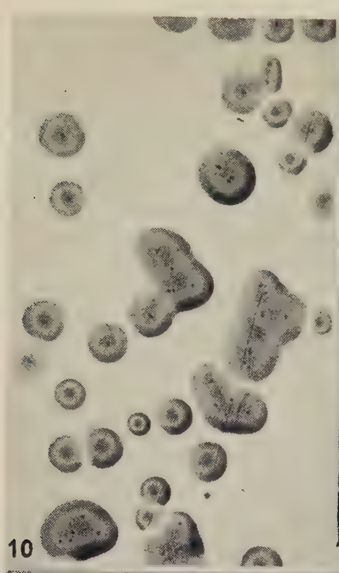
Plate 2



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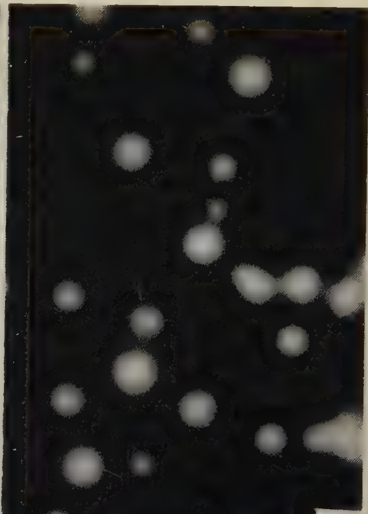

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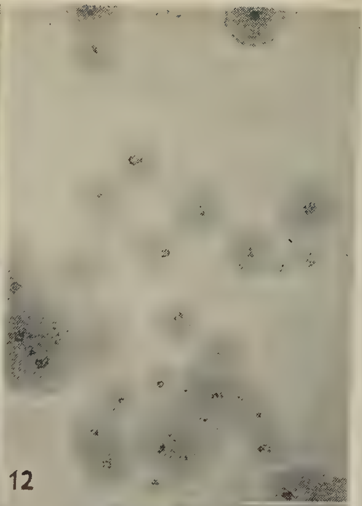

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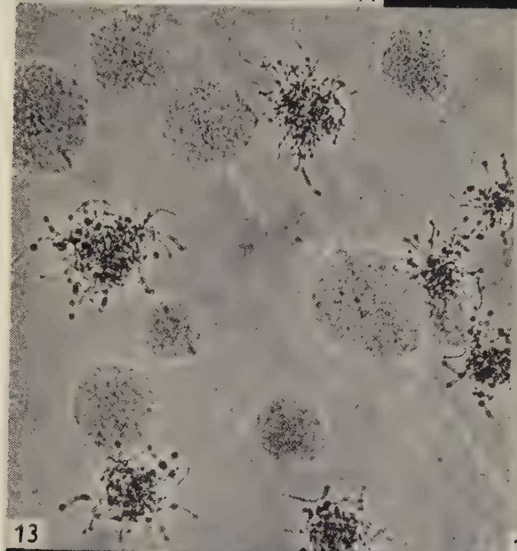

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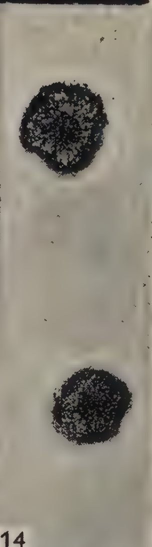

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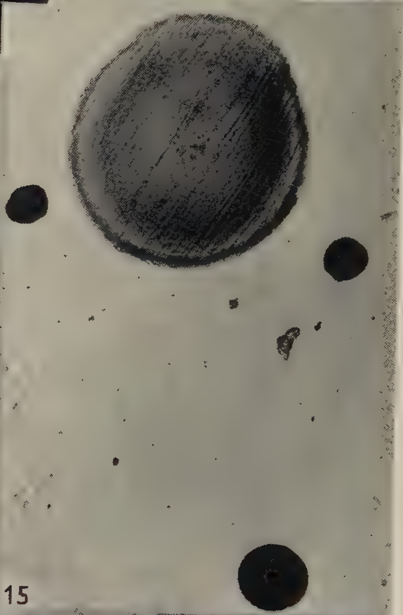

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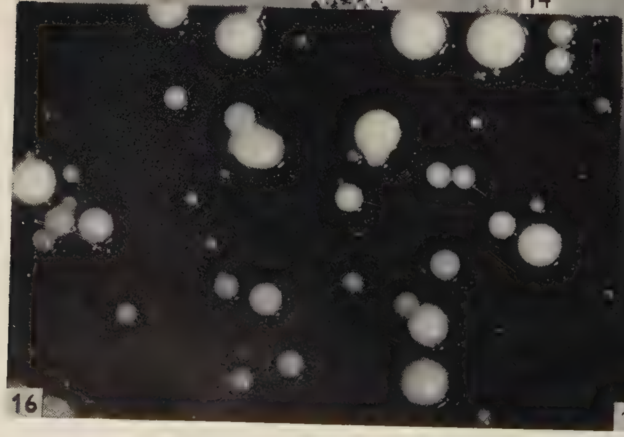

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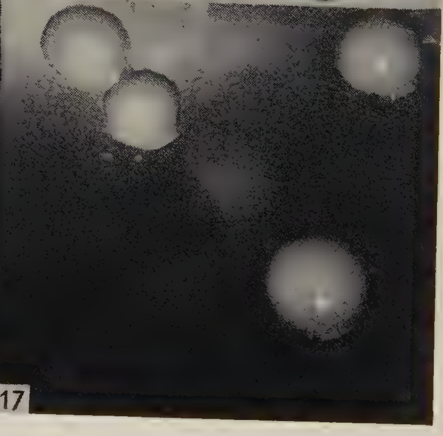

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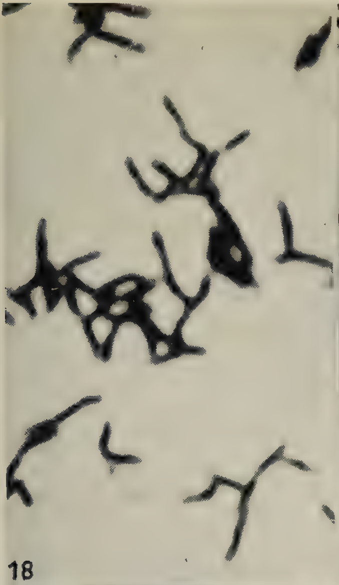

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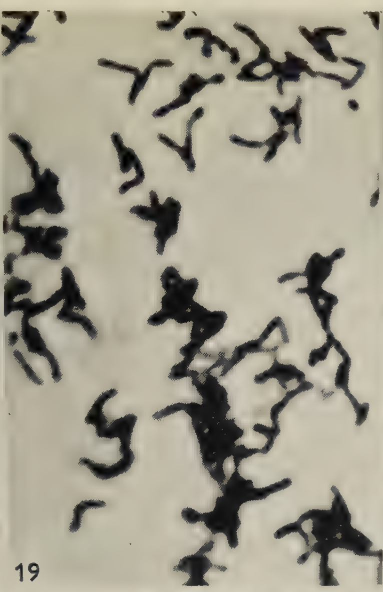

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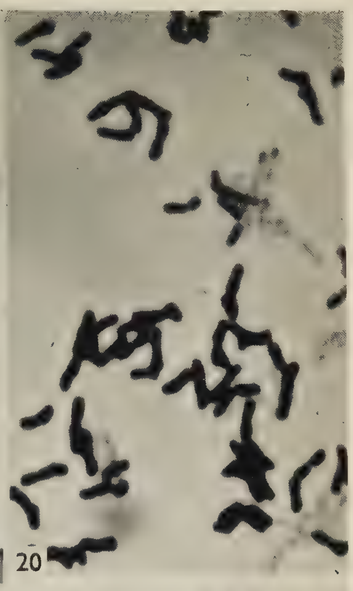

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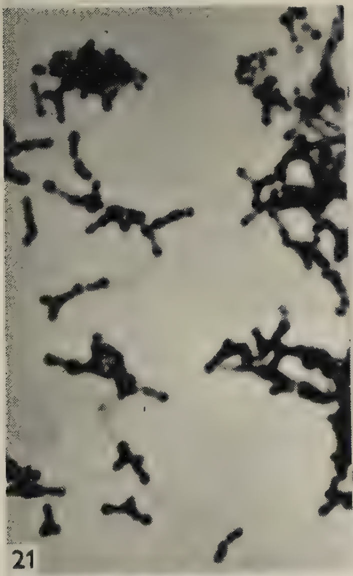

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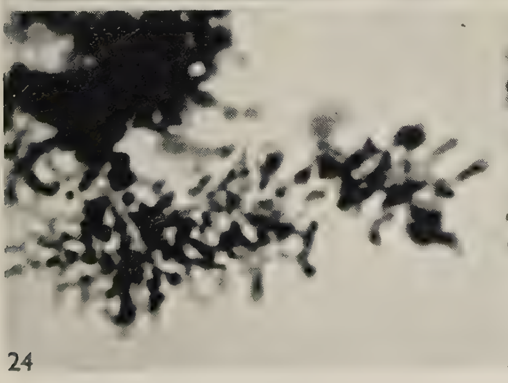

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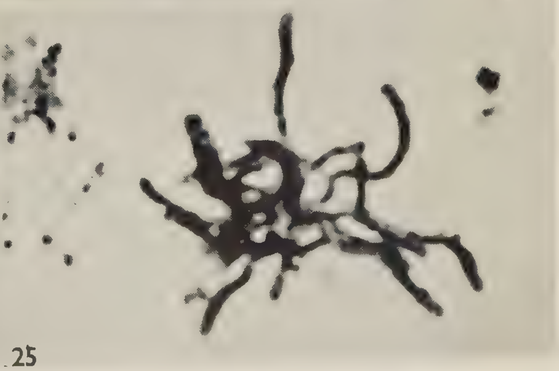

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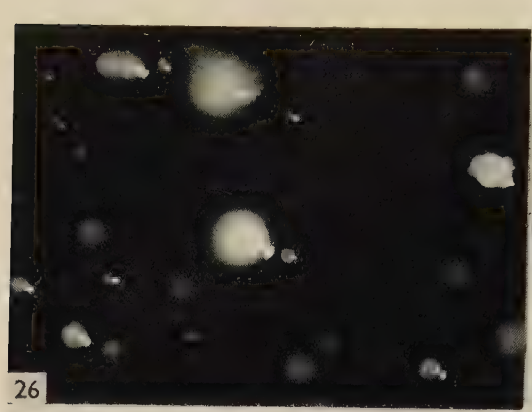
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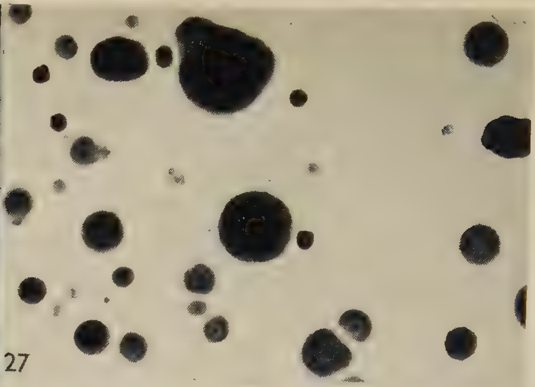
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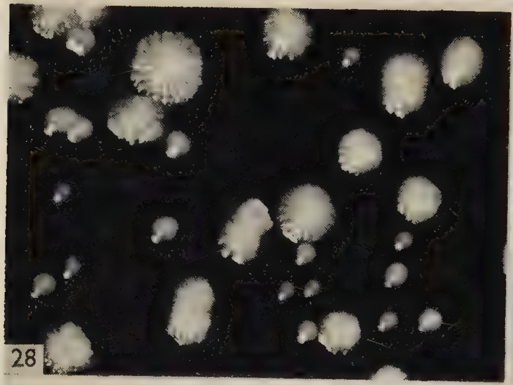
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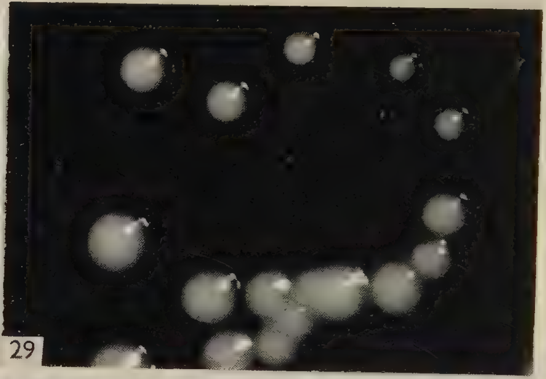
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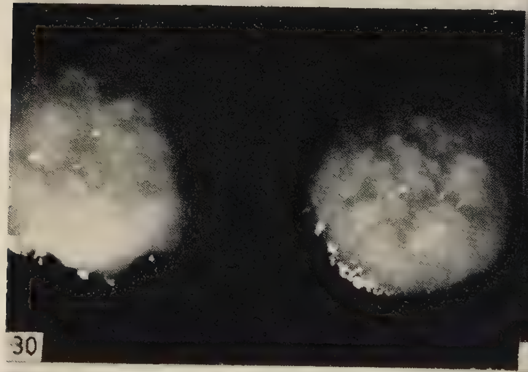
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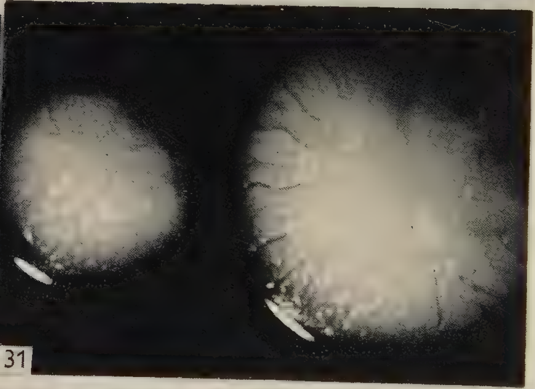
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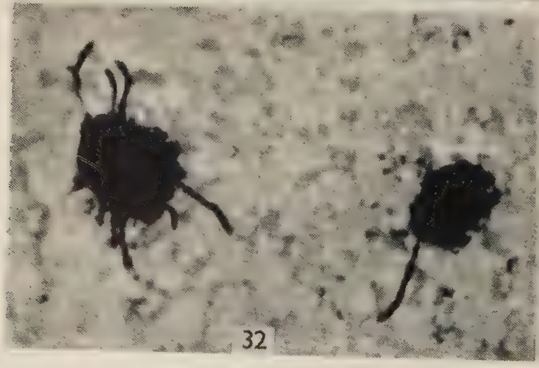
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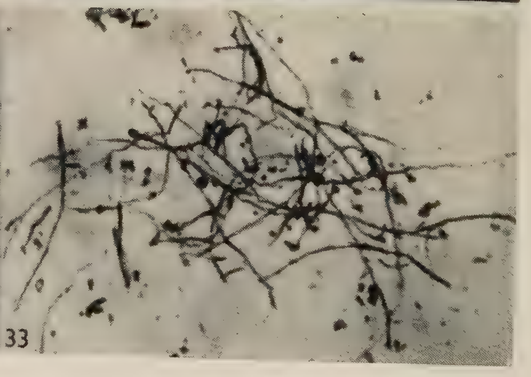
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EXPLANATION OF PLATES*

PLATE 1

Fig. 1. 'Sulphur' granule, case P1. (KOH mount.) × 115.

Fig. 2. 'Sulphur' granules showing 'clubs'. Case P1. (KOH mount.) × 678.

Fig. 3. 'Sulphur' granule, imbedded and sectioned. Case P1. Gram stain. × 450.

Fig. 4. *Actinomyces israelii*, strain OS131. Spider colonies. Brain heart infusion agar, transmitted light. × 300.

Fig. 5. *Actinomyces bovis*, P3S. Brain heart infusion agar, transmitted light. × 290.

Fig. 6. *A. bovis*, P3R. Brain heart infusion agar, transmitted light. × 171.

Fig. 7. *A. bovis*, P4S and P4R colonies. Isolation plate, Garrod's medium, oblique lighting. × 70.

Fig. 8. *A. bovis*, P4R. Rough colonies on isolation plate. Maintenance medium, transmitted light. × 70.

Fig. 9. *A. bovis*, P4R. Rough colony on maintenance medium, photographed under cover slip, transmitted light. × 950.

PLATE 2

Fig. 10. *Actinomyces bovis*, A11. Smooth colonies on brain heart infusion agar, transmitted and reflected light. × 13.

Fig. 11. *A. bovis*, A11. Smooth colonies on brain heart infusion agar, reflected light. × 13.

Fig. 12. *A. bovis*, P2S. Maintenance medium, transmitted and reflected light. × 70.

Fig. 13. *A. bovis*, case P2. Primary isolation on maintenance medium. Rough (spider) and smooth colonies, transmitted light. × 290.

Fig. 14. *A. bovis*, P2R. Later stage of development of spider colonies shown in fig. 13, transmitted light. × 170.

Fig. 15. *A. bovis*, case P2. Primary isolation on Garrod's medium showing relative size of three rough colonies and one smooth colony after 5 days incubation. Transmitted and reflected light. × 70.

Fig. 16. *A. bovis*, P2S. Seven-day colonies on maintenance medium, reflected light. × 13.

Fig. 17. *A. bovis*, P2R. Fourteen-day colonies on maintenance medium, reflected light. × 45.

PLATE 3

Fig. 18. *Actinomyces israelii*, 534. Branching bacillary elements, grown in maintenance broth. Gram stain. × 1100 enlarged to × 2200.

Fig. 19. *A. israelii*, OS131. Branching diphtheroid elements, grown in maintenance broth. Gram stain. × 1100 enlarged to × 2200.

Fig. 20. *Actinomyces bovis*, A12. Branching diphtheroid elements, grown in maintenance broth 24 hr. Gram stain. × 1100 enlarged to × 2200.

Fig. 21. *A. bovis*, A12. Branching diphtheroid elements, grown in maintenance broth 72 hr., showing metachromatic granules. Gram stain × 1100 enlarged to × 2200.

Fig. 22. *A. bovis*, P2S. Branching diphtheroid elements, grown in maintenance broth, 24 hr., showing amorphous cellular structures. Gram stain. × 1100 enlarged to × 2200.

Fig. 23. *A. bovis*, P2R. Fine branching bacillary elements of rough strain isolated from same cow as strain P2S. Maintenance broth, 4 days, Gram stain. × 1100 enlarged to × 2200.

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* The macrocolonies photographed were 3–5 days old.

Fig. 24. *A. bovis*, A11R. Young mycelial clump grown 24 hr. in maintenance broth with 0·05 % agar. Lactophenol-cotton blue mount. × 1100 enlarged to × 2200.

Fig. 25. *A. bovis*, A11R. Young mycelial colony, grown 24 hr. in maintenance broth with 0·05 % agar. Gram stain. × 1100 enlarged to × 2200.

PLATE 4

Fig. 26. *Actinomyces bovis*, A11. Maintenance agar, reflected light. Same microscopic field as in fig. 27. Colonies having high lights were considered as rough colonies. × 13.

Fig. 27. *A. bovis*, A11. Maintenance agar, transmitted light. Same microscopic field as in fig. 26. Colonies showing concentric rings of transmitted light were considered as smooth colonies. Opaque colonies were rough colonies. × 13.

Fig. 28. *A. bovis*, P3R. Stock culture, showing presence of smooth colonies. Maintenance agar, reflected light. Compare with fig. 6. × 13.

Fig. 29. *A. bovis*, P3S. Stock culture. Note absence of rough colonies. Maintenance agar, transmitted light. × 13.

Fig. 30. *A. bovis*, P3S. Effect of medium on macrocolony morphology. Thioglycollate agar, reflected light. × 29.

Fig. 31. *A. bovis*, P3S. Effect of medium on macrocolony morphology. Inoculum same as that in fig. 30. Maintenance agar, reflected light. × 29.

Fig. 32. *A. bovine*, P4R. Pus from hamster. Mycelial elements emanating from masses of cells. Gram stain. × 1100.

Fig. 33. *A. bovis*, P2S. Pus from hamster. Gram stain. × 900.

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The Adaptive Degradation of L-Histidine by *Paracolobactrum aerogenoides*

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SUMMARY

The characteristics of an induced enzyme system responsible for the degradation of L-histidine by a soil organism were studied. Induction could be achieved by exposing cells to L-histidine, D-histidine, urocanate, β-alanyl-L-histidine or L-histidyl-L-histidine, but not by exposure to a variety of other imidazoles. The kinetics of induction by L- and D-histidine differed considerably and the D-isomer was shown to be metabolized only very slowly by fully induced cells. Chloramphenicol and DL-*p*-fluorophenylalanine strongly inhibited enzyme synthesis, whilst a variety of purine and pyrimidine analogues were without effect. Nitrogen starvation of non-induced cells decreased but did not completely prevent the appearance of the enzyme system on induction with D-histidine. Non-induced cells possess a low level of 'basal' enzymes before exposure to an inducing substance.

INTRODUCTION

The pathway of L-histidine degradation has been studied in both animal tissues and bacteria. These studies led to the recognition of urocanic acid (β-imidazole acrylic acid) and glutamic acid as intermediates in histidine breakdown, and have been briefly reviewed by Tabor (1955) and Greenberg (1954). Tabor & Hayaishi (1952) studied the degradation of histidine by *Pseudomonas fluorescens*, and Tabor and his co-workers showed that extracts of this organism degraded L-histidine to ammonia, formate and glutamate; urocanate was implicated as an intermediate (Tabor, 1955). Magasanik & Bowser (1955) described similar experiments with *Aerobacter aerogenes*. The pathway of histidine breakdown in this organism resembled closely that found in *Pseudomonas fluorescens*, though differed in detail. Wickremasinghe & Fry (1954) demonstrated the formation of urocanate and glutamate during the breakdown of histidine by *Clostridium tetanomorphum*. Suda, Miyahara, Tomihata & Kato (1952) reported that the enzymes responsible for histidine degradation in a pseudomonad isolated from soil were inducible, and Magasanik (1953) presented similar evidence for *Aerobacter aerogenes*. Tristram (1953) presented a preliminary report of the induced breakdown of L-histidine by a soil organism. The purpose of this paper is to examine the factors affecting the induction in this organism.

METHODS

Organism. The organism used in this work was originally isolated from soil and given the number 232. It was subsequently identified by Dr M. E. Rhodes (Reading University) as a strain of *Paracolobactrum aerogenoides* (*Bergey's Manual*, 1957).

Chemicals. L- and D-histidine were obtained from Hoffman La Roche; urocanic acid, DL-*p*-fluorophenylalanine, DL-ethionine and β-2-L-thienylalanine from L. Light and Co. Colnbrook; purine and pyrimidine analogues from Sigma Chemical Co.; histamine and benzimidazole from British Drug Houses Ltd.; chloromycetin (chloramphenicol) from Parke, Davis and Co.; imidazole and 4:5 imidazole dicarboxylic acid from Eastman Kodak, and inorganic salts of 'Analar' quality from British Drug Houses Ltd. The Radiochemical Centre, Amersham, supplied [2-ring-^{14}C]-L-histidine, and β-(pyrazolyl-3)-alanine was prepared by Professor Dr R. Hüttel of Munich.

Media and cultivation. Unless otherwise stated the organism was grown on a medium containing 150 ml. 0·2 M-KH_2PO_4 with the following additions: glucose, 10·0 g.; NH_4Cl, 2·0 g.; $MgSO_4.7H_2O$, 0·1 g.; NaCl, 1·0 g.; $FeSO_4.7H_2O$, trace; N-NaOH to pH 7·2 (about 21 ml.) and Pyrex-distilled water to 1 l. Cultures were maintained as slopes on the above medium with the addition of 1·5 % (w/v) Difco agar and after incubation at 30° stored at 4°. Cells for metabolic experiments were grown in 75 ml. portions of liquid medium contained in 250 ml. Erlenmeyer flasks, inoculated with 2 ml. of an actively growing culture and incubated by shaking for 18 hr. at 30°. The organism was harvested by centrifugation and resuspended in 0·067 M phosphate buffer, pH 7·2. The rate of endogenous respiration of cells immediately after harvesting was high and, unless otherwise stated, suspensions were starved overnight in the same phosphate buffer in shaken flasks at 30°, whereupon the rate of endogenous respiration fell to a low and constant level. Adapted cells were obtained by shaking starved cells in 0·067 M-phosphate buffer (pH 7·2) containing 0·003 M-L-histidine at 30° and following oxygen absorption in a pilot experiment until the rate became constant, after which the cells were washed and resuspended in phosphate buffer. Adapted cells were also produced by growth in the presence of L-histidine, either replacing ammonium chloride as sole nitrogen source, or replacing both glucose and ammonium chloride as sole sources of carbon and nitrogen in the basal medium described above. The L-histidine oxidizing capacity of cells grown in this way varied widely according to the age of culture from which the cells were harvested. Adapted cells retained their full activity for at least 6 days on storage at $-10°$, though similar cells stored in the presence of chloramphenicol lost about 25 % of their activity on storage for the same period.

Growth and dry weight content of suspensions were determined turbidimetrically in a Hilger absorptiometer, using a suitable calibration curve. Toluene-treated cells were obtained by adding a few drops of redistilled toluene to suspensions of induced or non-induced cells in 0·067 M-phosphate buffer (pH 7·2), and shaking for 30 min. at 20°.

Cell-free enzyme preparations. Cell-free extracts were prepared by grinding a cell paste at 0° with Microid Polishing Alumina (supplied by Griffin and Tatlock Ltd.), in a cooled glass mortar (McIlwain, 1948). The preparation was extracted with cold 0·067 M-phosphate buffer (pH 7·2) containing 0·001 M reduced gluthathione (GSH) and centrifuged at 5° to remove unbroken cells, alumina particles and cell debris.

Chemical estimations. Ammonia was estimated by microdiffusion in Conway units (Conway, 1947), followed by nesslerization of the centre-well fluid, using the Nessler solution of Folin & Denis (1916). The estimation of α-amino nitrogen was carried out by measurement at 570 mμ of the ninhydrin colour developed by the method of Moore & Stein (1948), with L-histidine as standard. Histidine and glutamate were separated chromatographically, using *n*-butanol + acetic acid + water as solvent and 0·1 % (w/v) ninhydrin in 95 % ethanol for the detection of the spots. L-Glutamate was estimated quantitatively by the glutamic acid decarboxylase of *Clostridium welchii* B.W. 21 (Epps, 1945; Gale, 1945). The quantitative estimation of urocanic acid was carried out by measurement of the absorption at 277 mμ in a Unicam S.P. 500 spectrophotometer (Mehler & Tabor, 1953).

Radioactivity was measured in a proportional gas flow (methane) counter, using ground glass planchettes. The thickness of samples was such that self absorption was negligible.

Enzyme assays. Histidase (histidine α-deaminase, histidine deaminase) activity was determined by measurement of the urocanate formed at 30° by cell-free extracts previously heated to 80° for 15 min., followed by rapid cooling (Tabor & Mehler, 1955). Routine estimations of the activity of cells towards L-histidine were made by measuring the rate of oxygen uptake in the presence of 0·003 M-L-histidine in 0·067 M-phosphate buffer (pH 7·2) at 30°, by conventional manometric methods. Comparisons between the rate of oxygen uptake by intact cells and the rate of ammonia production from L-histidine by cell-free extracts of partially induced cells suggested that measurements of oxygen uptake yielded valid estimates of the amounts of L-histidine degrading enzymes. This conclusion was supported by the observation that glutamate, an intermediate in L-histidine degradation, was rapidly oxidized at equal rates by both induced and non-induced cells, suggesting that changes associated with induction were confined to enzymes active exclusively in the conversion of L-histidine to glutamate, and not with the subsequent oxidation of the glutamate formed.

RESULTS

The inducible nature of L-*histidine oxidation*

Organisms grown in basal medium and then starved in 0·067 M-phosphate buffer (pH 7·2), exhibited a low rate of oxygen uptake on the addition of L-histidine. However, the rate of oxygen absorption increased until it reached a high and constant value in about 4 hr. (Fig. 1). Under these conditions the Q_{O_2} increased from *c.* 7 to 50–55 and did not increase further in the presence of a higher initial L-histidine concentration or on the addition of a second quantity of the amino acid when oxidation of the first addition had ceased. Organisms grown in basal medium in which ammonium chloride was replaced by L-histidine (5 g./l.), or in which both glucose and ammonium chloride were replaced by L-histidine as sole source of carbon and nitrogen exhibited a high rate of oxygen absorption immediately on the addition of L-histidine to washed organisms in 0·067 M-phosphate (pH 7·2) (Fig. 1).

Non-induced organisms grown in basal medium lacked the ability to oxidize urocanate but adaptation to L-histidine also imparted the ability to oxidize urocanate. Suspensions of starved organisms also acquired ability to degrade urocanate when shaken with 0·003 M-urocanate and were able to oxidize L-histidine. Urocanate was never oxidized as rapidly as L-histidine by organisms induced with L-histidine or urocanate, due possibly to the failure of urocanate to penetrate readily into the cell. Glutamate was oxidized rapidly and at equal rates by unadapted organisms and by organisms exposed to either L-histidine or urocanate.

Effect of inhibitors. Addition of $2{\cdot}0\times10^{-4}$ M-2:4-dinitrophenol, $4{\cdot}0\times10^{-3}$ M-sodium azide, $5{\cdot}0\times10^{-3}$ M-DL-*p*-fluorophenylalanine or $5{\cdot}0\times10^{-2}$ M β-2-L-thienylalanine to non-induced organisms in presence of L-histidine under conditions described above, completely suppressed the adaptive response. The separate addition of 5-bromouracil, 6-amino-2-mercaptopurine sulphate, 2:6-diamino-purine sulphate or 5-fluorouracil, all at a concentration of 500 μg./ml., were without effect on the course of induction. At the same concentration 8-azaguanine was slightly inhibitory.

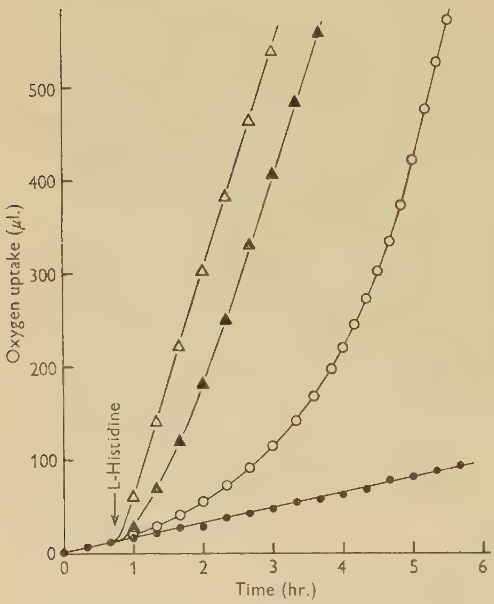


Fig. 1

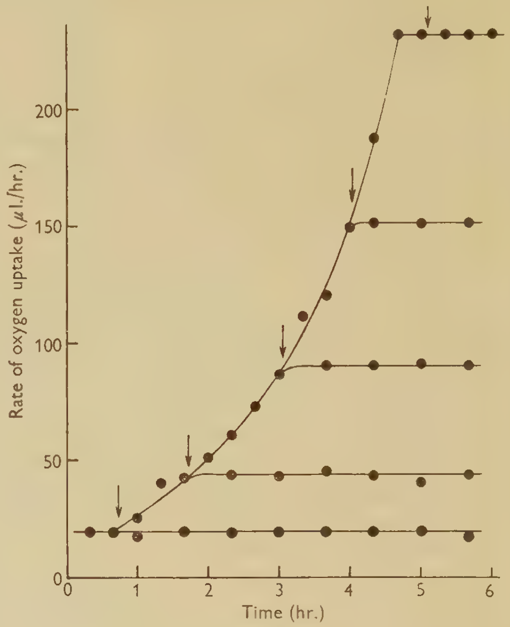


Fig. 2

Fig. 1. The effect of addition of 0·003 M-L-histidine on the oxygen uptake of cells grown in basal medium (○); basal medium containing L-histidine as sole source of nitrogen (▲); basal medium containing L-histidine as sole sources of carbon and nitrogen (△). Endogenous oxygen uptake of cells grown in basal medium (●). Warburg flasks contained 4 mg. dry wt. cells suspended in 0·067 M-phosphate buffer, pH 7·2.

Fig. 2. Effect of chloramphenicol on the rate of oxygen uptake in the presence of L-histidine. Two-arm Warburg flasks containing: (*m.c.*) 2 ml. suspension of non-induced cells (2 mg. dry wt. cells/ml.) in 0·067 M phosphate buffer, pH 7·2; (*s.b.* 1) 0·3 ml. 0·033 M L-histidine; (*s.b.* 2) 0·2 ml. chloramphenicol solution (625 μg./ml.); (*c.w.*) 0·2 ml. 10 % (w/v) KOH. L-Histidine added to all flasks after 40 min. Chloramphenicol added at times indicated by arrows.

The adaptive response of non-induced organisms to L-histidine was prevented by chloramphenicol at a concentration of 5 μg./ml., whereas 1000 μg./ml. did not inhibit the oxidation of L-histidine by induced organisms. Addition of chloramphenicol (50 μg./ml.) during the course of induction resulted in oxygen absorption continuing linearly at the rate reached at the moment of addition of the inhibitor (Fig. 2). Chloramphenicol was accordingly used for the prevention of further enzyme synthesis during the experiments to be described below.

Presence of a permeability barrier or enzyme inhibitor in non-induced cells. The effect of inhibitors suggests the synthesis of enzyme protein (or proteins) was involved during adaptation to L-histidine, though synthesis of a protein component of a transport mechanism responsible for the penetration of L-histidine into the cells might be involved. Cell-free extracts of induced cells, or toluene-treated induced cells, produced glutamate and ammonia from L-histidine, whereas similar treatment of non-induced cells yielded almost inactive preparations (Table 1). Preparations of non-induced cells produce small amounts of ammonia and glutamate owing to the presence of 'basal' enzymes in such cells.

Table 1. *Production of ammonia and glutamate from* L-*histidine by extracts and toluene-treated induced and non-induced organisms*

Incubation mixtures consisted of cell extract in 0·067 M-phosphate buffer (pH 7·2), containing 0·001 M-GSH or toluene-treated cell suspension (5 mg. dry wt./ml.) in 0·067 M-phosphate buffer (pH 7·2), together with 6·0 μmole. L-histidine/ml. Incubation at 30° for 2 hr. (toluene-treated cells) or for 3 hr. (cell-free extracts). All figures corrected for blank values obtained from control preparations without added L-histidine.

		Residual L-histidine (μmole./ml.)	Ammonia formed (μmole./ml.)	L-Glutamate formed (μmole./ml.)
Cell extracts	induced cells	1·8	8·1	3·9
	non-induced cells	5·7	0·2	Trace*
Toluene-treated cells	induced cells	0	11·8	5·9
	non-induced cells	5·5	0·8	Trace*

* Preparations from non-induced cells produced insufficient L-glutamate to estimate by the decarboxylase method, but a trace of glutamate was detected chromatographically.

The possibility that non-induced cells possess the enzymes responsible for L-histidine degradation but contain an inhibitor which is in some way removed on the addition of inducer, appeared unlikely since the addition of a cell-free extract of non-induced cells had no inhibitory effect on the production of ammonia from L-histidine by a cell-free extract of induced cells.

The occurrence of 'basal' enzyme in non-induced cells. The addition of chloramphenicol to thick suspensions of non-induced cells was without significant effect on the rate of endogenous respiration, but the further addition of L-histidine resulted in a slight, but reproducible, stimulation of oxygen uptake (Table 3). This observation, under conditions where enzyme synthesis was prevented by chloramphenicol, suggests the presence of some 'basal' enzyme in non-induced cells. The oxidation of L-histidine by induced cells results in the formation of CO_2. Exposure of non-induced cells to (2-ring-^{14}C)-L-histidine in the presence of chloramphenicol resulted in the appearance of $^{14}CO_2$ in the respiratory CO_2 (Fig. 3). Thus non-induced cells under conditions precluding induced enzyme synthesis possessed the capacity to liberate the C-2 of the imidazole ring as CO_2. The rate of liberation of $^{14}CO_2$ was equivalent to 2533 counts/min./flask/hr. A suspension of fully induced cells of similar cell density liberated 125,685 counts/min./flask/hr. From these values the 'basal' enzymes in non-induced cells are present to the extent of *c.* 2 % of the values found in fully induced cells.

Figure 3 also shows that there was no lag in the liberation of $^{14}CO_2$ from

(2-ring-^{14}C)-L-histidine, an observation which supports the view that non-induced cells are permeable to L-histidine, without the prior synthesis of a transport mechanism (or permease).

The ability of imidazoles to act as inducing substances

A variety of imidazoles other than L-histidine were tested for their ability to induce the formation of enzymes responsible for the degradation of L-histidine. Suspensions of non-induced cells were exposed to compounds for 4 hr., further enzyme synthesis being prevented by the addition of chloramphenicol. After washing and resuspending the cells in 0·067 M-phosphate buffer (pH 7·2) containing chloramphenicol (50 μg./ml.), the ability of suspensions to oxidize L-histidine was measured by determining the rate of oxygen uptake in the presence of L-histidine. Examination of the results of such an experiment (Table 2) reveals that urocanic acid, D-histidine, L-histidyl-L-histidine and β-alanyl-L-histidine were capable of acting as inducers, whereas a variety of other imidazoles were devoid of activity. The single pyrazole tested, namely β-(pyrazolyl-3)-alanine was unable to induce the synthesis of L-histidine degrading enzymes. The enzyme activities induced in the presence of L-histidyl-L-histidine, β-alanyl-L-histidine, urocanate and D-histidine were in all instances considerably lower than that induced by an identical molar concentration

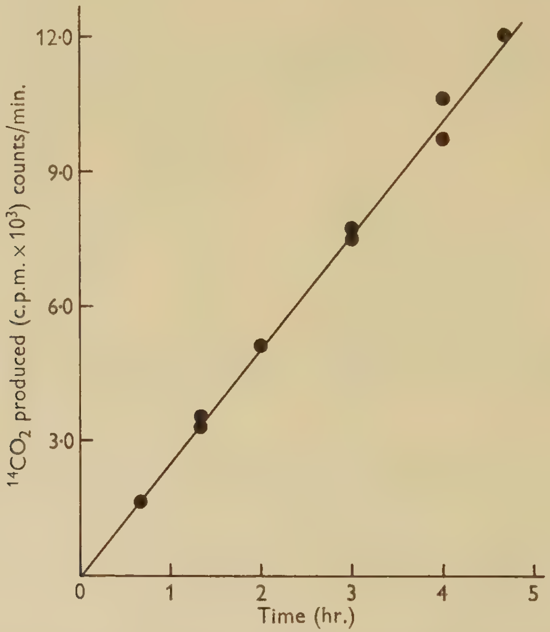


Fig. 3. Liberation of [^{14}C]carbon dioxide from [2-ring-^{14}C]-L-histidine by non-induced cells. Two-arm Warburg flasks contained: (*m.c.*) 2 ml. cell suspension (3·8 mg. dry wt./ml.) in 0·067 M-phosphate (pH 7·2); 0·2 ml. chloramphenicol solution (625 μg./ml.); (*s.b.* 1) 0·3 ml. 0·033 M [2-ring-^{14}C]-L-histidine (264,300 counts/min./flask); (*s.b.* 2) 0·2 ml. 5 N-H_2SO_4 added to stop the reaction and to liberate any CO_2 bound by the phosphate buffer; (*c.w.*) 0·2 ml. 10 % (w/v) KOH. Radioactivity of CO_2 produced was estimated by quantitative removal of centre well fluid and counting as K_2CO_3.

of L-histidine. It appeared likely that induction by the two dipeptides was due to hydrolysis to free histidine, but this point was not investigated further.

The possibility that induction by D-histidine was due to contamination by the L-isomer was prevented by using a preparation of the D-isomer freed of L-histidine by previously shaking an aqueous solution of commercial D-histidine with a thick suspension of fully induced organisms for 1 hr. to destroy any L-histidine, followed by centrifugation and precipitation of the D-isomer by addition of absolute ethanol. This product did not support the growth of *Streptococcus faecalis R* or *Leuconostoc mesenteroides* P-60 in media suitable for the micro-biological assay of L-histidine (Henderson & Snell, 1948).

Table 2. *The ability of various imidazoles to induce the formation of* L-*histidine degrading enzymes*

Incubation mixtures contained 10 ml. cell suspension (2 mg. dry wt./ml.) in 0·067 M-phosphate buffer (pH 7·2) and 0·003 M-inducing substance. After shaking 4 hr. at 30° chloramphenicol added to 50 μg./ml.; cells washed with 0·067 M-phosphate buffer (pH 7·2) containing 50 μg./ml. chloramphenicol and resuspended in the same solution. The amount of enzyme(s) synthesized measured as the rate of oxygen uptake in the presence of 0·003 M-L-histidine. The endogenous respiration rate (27 μl. O_2 absorbed/hr.) has been subtracted from all values.

Addition	μl. O_2/hr.	Addition	μl. O_2/hr.
L-Histidine	205	4:5-Imidazole dicarboxylic acid	6
Urocanic acid	136	Imidazole	5
D-Histidine	93	Histamine	−1
L-Histidyl-L-histidine	95	β-(Pyrazolyl-3)alanine	3
β-Alanyl-L-histidine	88	None	4
Benzimidazole	8		

Metabolism of D-*histidine*

The use of thick suspensions of non-induced organisms revealed a higher rate of oxygen uptake in the presence of D-histidine compared with the endogenous respiration rate. The further addition of chloramphenicol to suppress enzyme synthesis in presence of D-histidine resulted in a decrease in the rate of oxygen uptake to a value very close to the endogenous rate (Table 3). There is good evidence that the respiration rate of many systems is controlled by the concentration of phosphate acceptors and since the addition of D-histidine initiates enzyme synthesis this may increase the concentration of phosphate acceptors and hence increase the rate of respiration (Chance & Williams, 1956). The addition of D-histidine to induced organisms resulted in significant stimulation of the rate of oxygen uptake compared with the rate observed in control organisms. Further addition of chloramphenicol had little or no effect (Table 3). Comparison of rates of oxygen uptake by induced organisms in presence of D- or L-histidine reveals that, after subtraction of the endogenous rate of oxygen absorption, the rate of oxidation of the D-isomer was about 10 % of the L-histidine oxidation rate; D-histidine did not inhibit the oxidation of L-histidine by induced organisms at D:L ratios as high as 25:1.

The formation of ammonia from L- and D-histidine by thick suspensions of intact induced organisms and toluene-treated induced organisms is shown in Fig. 4. Ammonia production from the D-isomer was considerably less than from L-histidine.

The addition of L-histidine to toluene-treated induced organisms resulted in the accumulation of virtually quantitative amounts of glutamate (Table 1 and Fig. 4). In several experiments involving the addition of the D-isomer to such organisms no significant glutamate production was detected above the trace amounts found chromatographically in control organisms to which water had been added.

Extracts of induced organisms yielded ammonia from L-histidine, but not from the D-isomer (Fig. 5). Tabor & Mehler (1955) showed that urocanase activity of extracts of *Pseudomonas fluorescens* could be eliminated by heating the cell-free extract to 80° for 15 min. The histidase activity of heated extracts was unimpaired and resulted in the accumulation of urocanate from L-histidine. Figure 5 shows that

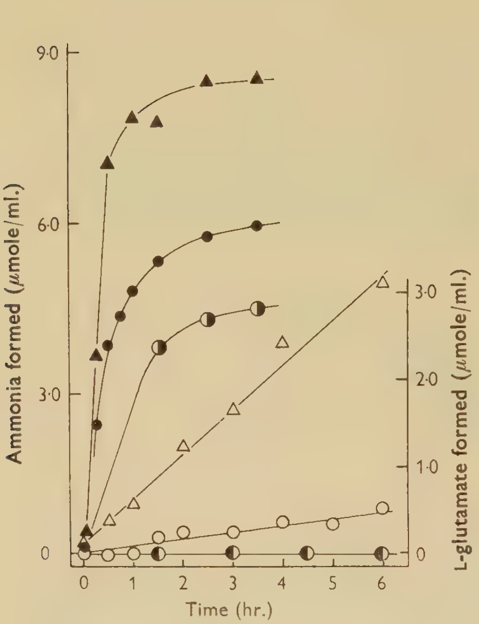


Fig. 4

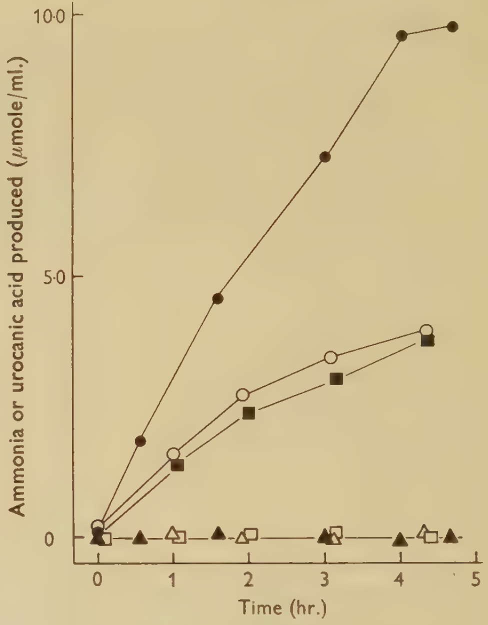


Fig. 5

Fig. 4. Production of ammonia and L-glutamate by intact and toluene-treated induced cells. Incubation mixtures, contained in 250 ml. Erlenmeyer flasks shaken at 30°, consisted of intact or toluene-treated cell suspensions (7·3 mg. dry wt. cells/ml.) in 0·067 M-phosphate (pH 7·2) to which 3 μmole L- or D-histidine/ml. was added at 0 hr. Intact cells: ▲—▲, ammonia production in the presence of L-histidine; △—△, ammonia production in the presence of D-histidine. Toluene-treated cells: ●—●, ammonia production in the presence of L-histidine; ○—○, ammonia production in the presence of D-histidine; ◑—◑, glutamate production in the presence of L-histidine; ◐—◐, glutamate production in the presence of D-histidine. All values corrected for control flasks containing an equivalent volume of water in place of L- or D-histidine solution.

Fig. 5. Production of ammonia and urocanate by extracts and heated extracts of induced cells. Incubation mixtures, maintained at 30°, consisted of cell extract or heated extract in 0·067 M-phosphate buffer (pH 7·2), containing 0·001 M-GSH to which 5 μmole. L- or D-histidine was added at 0 hr. Cell extract: ●—●, ammonia production in the presence of L-histidine; ▲—▲ ammonia production in the presence of D-histidine. Heated cell extract: ○—○, ammonia production in the presence of L-histidine; △—△, ammonia production in the presence of D-histidine; ■—■, urocanate production in the presence of L-histidine; □—□, urocanate production in the presence of D-histidine. All values corrected for control extracts containing an equivalent volume of water in place of L- or D-histidine solution.

heated extracts of organism 232 also produced ammonia and urocanate from L-histidine, but no increase in either ammonia or urocanate could be detected in the presence of D-histidine. Thus D-histidine was apparently metabolized by the organism studied, though the rate of metabolism was very much slower than that of the L-isomer. Furthermore, the failure to demonstrate ammonia and urocanate formation from D-histidine by extracts able to produce both these substances from L-histidine suggests that the oxygen absorption observed when the D-isomer was added to induced organisms was the result of a different metabolic pathway from that responsible for the degradation of L-histidine.

Table 3. *Rates of oxygen uptake by induced and non-induced organisms in the presence of L-histidine, D-histidine and chloramphenicol*

After growth and starvation (see Methods) cells were suspended in 0·067 M-phosphate buffer (pH 7·2), and half of the suspension was induced with L-histidine. The remainder of the suspension was retained as non-induced organisms. Both portions were washed, re-suspended in phosphate buffer and samples containing 16 mg. dry wt. organisms placed in Warburg flasks, together with additions indicated below. Values in the table represent μl. O_2 utilized/hr.

Additions	Non-induced	Induced
Nil (endogenous)	119 ± 0·3	114 ± 1·8
Chloramphenicol (50 μg./ml.)	116 ± 1·3	110 ± 2·3
0·003 M-D-histidine	164 ± 2·7	181 ± 2·5
0·003 M-D-histidine + chloramphenicol (50 μg./ml.)	118 ± 0·3	188 ± 1·7
0·003 M-L-histidine + chloramphenicol (50 μg./ml.)	123 ± 1·9	920*
	—	9·6†

Figures represent the mean ± standard error of the mean, based on 4 determinations.

* Values obtained by calculation from suspensions of induced organisms diluted with 0·067 M-phosphate buffer, pH 7·2.

† Rate of oxidation of D-histidine expressed as percentage of the rate of oxidation of L-histidine, after correction for the endogenous oxygen absorption.

The kinetics of induction

The kinetics of induction, together with the factors affecting the induction process, were studied by taking samples of organisms exposed to inducer into centrifuge tubes containing chloramphenicol solution to give a final concentration of 50 μg. chloramphenicol/ml., centrifuging, washing and resuspending the organisms in sufficient 0·067 M-phosphate buffer (pH 7·2) containing chloramphenicol to give 2 mg. dry wt. cells/ml. suspension. The amount of induced enzyme or enzymes synthesized was measured by the determination of the rate of oxygen uptake by 2 ml. samples of these washed suspensions in the presence of 0·003 M-L-histidine.

Induction by L-histidine. The kinetics of enzyme induction in a washed cell population exposed to L-histidine is shown in Fig. 6. Increasing the initial concentration of the inducer from 0·003 M- to 0·01 M-L-histidine did not affect the maximal level of enzymic activity, but delayed the onset of the subsequent decrease in activity, which was correlated with the disappearance of the inducer.

Induction by D-histidine. When D-histidine was added to a washed suspension of the organism enzymes capable of degrading the L-isomer were formed, but the kinetics of the induction differed from those observed when L-histidine was used as the inducing agent. Enzyme production was linear and the maximum enzyme value was not reached until 6–7 hr. after addition of the inducer (Fig. 7). Furthermore, the final enzyme value attained was lower than that reached by organisms induced with L-isomer and was stable for at least 12 hr. This stability was shown to be due to continued presence of D-histidine, whereas under the conditions of the experiments induction by L-histidine was followed by a 'decay' of enzyme activity following the rapid exhaustion of the inducer. Within the limits of accuracy of the method used for measurement of enzyme activity the formation of enzyme commenced immediately on the addition of inducer.

Organisms induced with D-histidine oxidized not only L-histidine, but also urocanic acid. In a typical experiment a suspension of organisms induced with D-histidine oxidized L-isomer at a rate equivalent to the uptake of 90 μl. O_2/hr. and in the presence of urocanate a sample of the same suspension used 63 μl. O_2/hr. The significance of

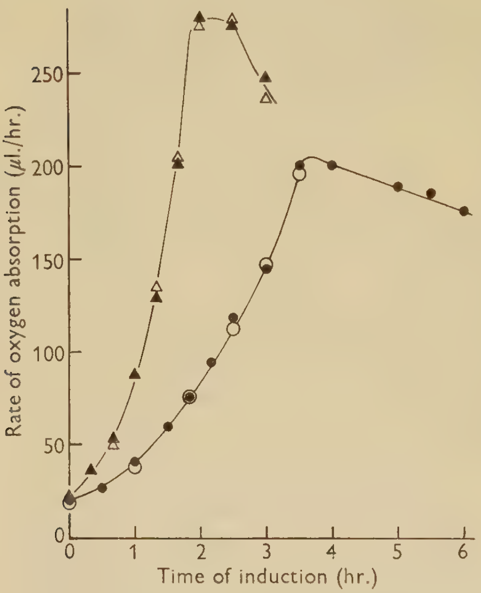


Fig. 6

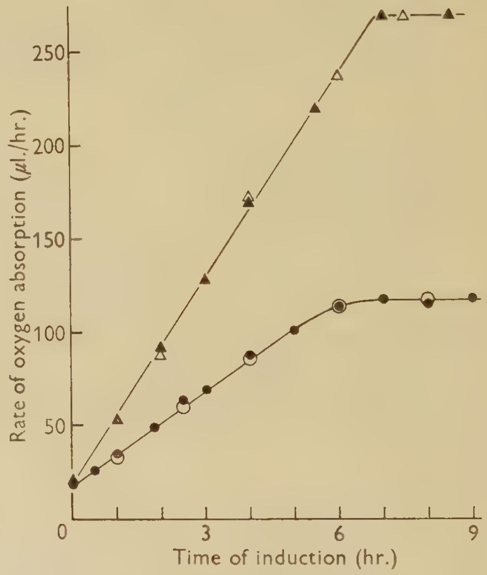


Fig. 7

Fig. 6. The induction of L-histidine degrading enzymes by L-histidine in the presence and absence of sodium succinate and ammonium chloride. Induction mixtures consisted of 120 ml. of cell suspension (2 mg. dry wt. cells/ml.) in 0·067 M-phosphate buffer (pH 7·2); (●); cell suspension + 0·02 M sodium succinate (▲); cell suspension + ammonium chloride (100 μg./ml.) (○); cell suspension + 0·02 M-sodium succinate + ammonium chloride (100 μg./ml.) (△). All flasks received, as inducer, 0·003 M-L-histidine at 0 hr. At intervals 8·0 ml. samples were withdrawn into centrifuge tubes containing sufficient chloramphenicol solution, to give a final concentration of 50 μg. chloramphenicol/ml. After washing in 0·067 M-phosphate buffer (pH 7·2) containing 50 μg. chloramphenicol/ml. the pellets were resuspended in the same solution to give 2 mg. dry wt. cells/ml. and duplicate 2 ml. samples placed in Warburg flasks. Enzyme synthesis was measured as the rate of oxygen uptake in the presence of 0·003 M-L-histidine. Endogenous respiration rates were obtained from a Warburg flask containing water in place of L-histidine. Each point represents the average of duplicate determinations. Endogenous respiration rate (23 μl. O_2/hr.) not subtracted.

Fig. 7. The induction of L-histidine degrading enzymes by D-histidine in the presence and absence of sodium succinate and ammonium chloride. Details and symbols as in Fig. 6, except that induction was accomplished with 0·003 M-D-histidine.

this observation will be discussed later (see Discussion). In all experiments described the initial inducer concentration was 3·33 μmole./ml. Under the conditions outlined above, using D-histidine as inducer, the induction mechanism appeared to be saturated and the maximum rate of enzyme synthesis achieved at a concentration of 2·0 μmole, D-histidine/ml.

The effect of succinate and ammonium chloride on induction. The difference in final enzyme values after induction with L- and D-histidine (Figs. 6 and 7) was consistently observed in a number of experiments.

This difference may be accounted for by exhaustion of endogenously available nitrogen sources, respiratory substrate, carbon for synthetic purposes (or a combination of these factors) in organisms induced with D-histidine, whereas in those induced with the L-isomer these reserves may be replenished from the products of L-histidine degradation. The effects of adding 0·02 M-sodium succinate and ammonium chloride (100 μg./ml.) on the development of induced enzyme activity, with L- and D-histidine as inducers are shown in Figs. 6 and 7. Addition of succinate resulted in an increase in the rate of enzyme formation and the final values of enzymic activity attained was higher than the corresponding maxima observed in the absence of succinate. Furthermore, in the presence of succinate, induction by D-histidine led to a final enzymic activity identical with that observed when organisms were induced by L-isomer. The addition of ammonium chloride (100 μg./ml.) was without effect on either the rate or final value of enzyme production whether organisms were induced with L- or D-histidine, either in the presence or absence of succinate.

Table 4. *Effect of nitrogen starvation on synthesis of* L-*histidine degrading enzymes*

Nitrogen-starved organisms prepared as described in the text were suspended in 0·067 M-phosphate buffer (pH 7·2); (2·18 mg. dry wt. cells/ml.) containing 0·003 M-D-histidine. After induction for periods indicated below, during which flasks were shaken at 30°, chloramphenicol (50 μg./ml.) added and organisms washed in 0·067 M-phosphate (pH 7·2) containing 50 μg. chloramphenicol/ml. and resuspended in the same solution. Enzyme synthesis was measured as the rate of oxygen uptake in the presence of 0·003 M-L-histidine. Results expressed as μl. oxygen utilized/hr.

	Time of induction (hr.)		
	0	3·0	6·0
Type I organisms: shaking in nitrogen-free and nitrogen-rich medium after growth in normal medium			
Nitrogen-deficient	41(−1)	69(33)	95(62)
Nitrogen-rich	31(0)	81(46)	116(83)
Type II organisms: growth in nitrogen-deficient and nitrogen-rich medium			
Nitrogen-deficient	38(2)	48(19)	62(39)
Nitrogen-rich	29(3)	84(60)	97(73)

Figures in parentheses represent values corrected for endogenous respiration rates.

The effect of nitrogen starvation

Nitrogen-starved organisms were obtained in two ways. Type I organisms were harvested after growth in normal medium, washed and resuspended aseptically in normal medium from which the nitrogen source was omitted, and shaken for 6 hr. at 30°. Control organisms were resuspended in medium containing normal amounts of ammonium chloride (see Methods). Type II organisms were obtained by growth organisms in medium containing a limiting amount of ammonium chloride (300 mg./l medium). Growth ceased due to the exhaustion of nitrogen supply and enzyme synthesis by these organisms was compared with control cells grown on normal medium. On addition of D-histidine both Type I organisms and Type II organisms were able to synthesize induced enzyme, though after a 6 hr. induction period Type I organisms had only 75 % of the activity of 'normal' ones and Type II organisms developed enzyme equivalent to 53 % of that of 'normal' organisms (Table 4). The endogenous rates of respiration of both types of nitrogen-starved organisms was high, presumably due to assimilation of carbon from the medium in the absence of exogenous nitrogen sources.

DISCUSSION

The synthesis of histidase and urocanase (and possibly other enzymes involved in the conversion of L-histidine to glutamate) has been shown to be inducible in *Paracolobactrum aerogenoides* (strain 232). The adaptive pattern in this organism closely resembles that described in a pseudomonad (Suda *et al.* 1952). The formation of urocanate, glutamate and ammonia by extracts of induced organisms and toluene-treated organisms (Figs. 4 and 5) suggests that the pathway of L-histidine degradation is similar to that observed in *Pseudomonas fluorescens* and *Aerobacter aerogenes* (Tabor, 1955; Magasanik & Bowser, 1955).

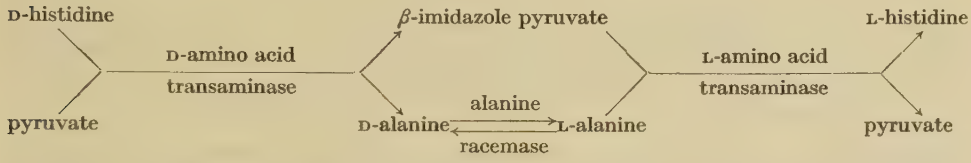
Competent inducers, structurally related to the substrates of induced enzymes, though incapable of acting as substrates have been described for a variety of bacterial systems (Monod, Cohen-Bazire & Cohn, 1951; Buttin, Cohen-Bazire & Monod, 1956; Duerksen & Halvorson, 1959). The failure to detect urocanate and glutamate formation from D-histidine by extracts of organism 232 supports the view that the D-isomer is behaving as a 'non-metabolizable' inducer. Addition of D-histidine to fully induced organisms does, however, lead to slight stimulation of the rate of oxygen uptake and ammonia production (Table 3 and Fig. 4).

Kallio & Larson (1955), studying the methionine racemase of a pseudomonad, and Gunsalus, Stanier & Gunsalus (1953), studying the mandelic acid racemase of *Pseudomonas fluorescens*, described the induction by the D-isomer of an enzyme attacking the corresponding L-isomer. Both these racemases differ from the histidine oxidation system since both isomers are substrates and are attacked at comparable rates. Shilo (1957) and Shilo & Stanier (1957) described a strain of soil pseudomonad capable of forming three stereospecific dehydrases active against *d*-, *l*- and *meso*-tartrates (see Shilo *loc. cit.* for configurational notation). The induction of each dehydrase was also highly stereospecific, so that only the isomer on which the organism had been grown was oxidized without a lag. La Rivière (1956) recorded similar observations with *P. putida*, but a situation at variance with these results has been described (Martin & Foster, 1957).

The induction pattern exhibited by D-histidine in our *Paracolobactrum* may be explained by the slow conversion of the D-histidine to the L-isomer, followed by sequential induction of urocanase by the urocanate produced from L-histidine by the action of histidase. Alternatively, since urocanate induces the formation of both histidase and urocanase, the conversion of D-histidine to urocanate by histidase would account for the experimental observations. This explanation seems unlikely, since the available evidence suggests that histidase does not attack the D-isomer.

Lastly both isomers of histidine, together with urocanate, may be converted to a compound which is responsible for the actual induction of the enzymes, or the induction site may be characterized by relatively poor specificity and capable of being activated by both isomers of histidine and by urocanate.

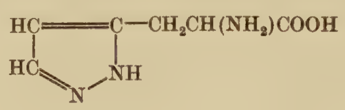
Three possible mechanisms by which D-histidine may be converted to the L-isomer may be discussed. First, the organisms may contain a histidine racemase comparable with the alanine racemase of *Streptococcus faecalis* and other bacteria (Wood & Gunsalus, 1951; Stewart & Halvorson, 1953; Marr & Wilson, 1954), the methionine racemase of a pseudomonad (Kallio & Larson, 1955) or the glutamate racemase of *Lactobacillus arabinosus* (Narrod & Wood, 1952). Secondly, D-histidine may be attacked by a D-amino acid oxidase, yielding β-imidazole pyruvate which, by transamination, could yield L-histidine. Finally, the D-isomer may be converted to the L-isomer by the successive actions of a D-amino acid transaminase, alanine racemase and L-amino acid transaminase as follows:



Thorne & Molnar (1955) demonstrated in some members of the genus *Bacillus* the presence of one or more enzymes capable of catalysing transaminations between pyruvate and some D-amino acids, including D-histidine. That D-histidine can be inverted in the animal body was demonstrated by Conrad & Berg (1937) who studied the growth of rats (for which histidine is an essential amino acid) on a diet containing the D-isomer as source of histidine. Analysis of carcasses showed an increase in histidine content and the histidine isolated was virtually pure L-histidine. Reviewing the utilization of D-amino acids by animals Berg (1959) suggested that the mechanism of this inversion consists of conversion to the α-keto analogue followed by re-amination by transamination.

Extracts of paracolobactrum 232, heat-treated to destroy urocanase activity (Tabor & Mehler, 1955), produced ammonia and urocanate from L-histidine, but no detectable amounts from the D-isomer (Fig. 5), and suggesting that the histidase of this organism is inactive against D-histidine. This observation is in agreement with that of Edlbacher & Kraus (1930) who stated that D-histidine is not attacked by animal histidase. These observations suggest that the D-isomer is metabolized by a route different from that of L-histidine, but do not exclude the possibility of the occurrence of a histidine racemase since this enzyme may be labile to the extraction procedures used, or more especially to the heat treatment necessary for the destruction of urocanase activity. The oxygen absorption and ammonia production observed on the addition of D-histidine to organism 232 (Table 3 and Fig. 4) may be associated with the activity of a D-amino acid oxidase. Most D-amino acid oxidases of animal tissues and most micro-organisms so far studied attack D-histidine and other basic amino acids very slowly (review by Krebs, 1951). However, Stumpf & Green (1946) reported that extracts of *Proteus vulgaris* and *P. morganii* oxidized D-histidine. No figures were quoted but only three other D-isomers (alanine, phenylalanine and phenylaminobutyrate) were attacked more rapidly than D-histidine.

The organism studied in the present investigation may contain such an oxidase of low activity, though apart from the very slow oxidation of D-histidine (Table 3) the organism oxidized D-phenylalanine but failed to oxidize the D-isomers of valine, leucine, isoleucine, methionine or tryptophan. The failure of histamine and the ability of urocanic acid (β-imidazole acrylic acid) to induce suggests that the α-COOH group (of histidine) must remain intact for activity. More striking is the observation that β-(pyrazolyl-3)-alanine (I), differing from histidine (II) only in that the $>$CH and =N— at the 2 and 3 positions, respectively, of the imidazole ring, are transposed, lacked inducing activity.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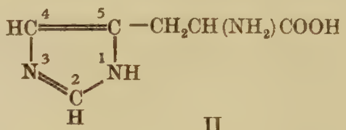
II

Pollock (1957) demonstrated that non-induced *Bacillus cereus* contained small levels of 'basal' penicillinase and the study of other induced enzymes for which a sufficiently sensitive assay exists has usually revealed the presence of small amounts of enzyme even in non-induced cells. Exposure of non-induced Paracolobactrum 232 to L-histidine under conditions precluding enzyme synthesis led to the liberation of the C-2 of the imidazole ring as CO_2 (Fig. 3). Furthermore toluene-treated non-induced cells and extracts of them produce small amounts of ammonia and glutamate from L-histidine (Table 1). It seems reasonable to assume that the mechanism of the production of these substances is identical with that found in induced organisms and that non-induced organisms contain small quantities of the enzymes formed on induction by histidine. The data presented in Table 1 also support the view that non-induced organisms lack at least one of the enzymes responsible for L-histidine breakdown and that the adaptive process does not involve the synthesis of an inducible transport mechanism (or permease) of the type described in other bacterial systems (Rickenberg, Cohen, Buttin & Monod, 1956; Green, 1956; Pardee, 1957). This conclusion is also supported by the fact that there was no lag in the evolution of $^{14}CO_2$ from (2-ring-^{14}C)-L-histidine by non-induced organisms, an observation which suggests that these are permeable to L-histidine (Fig. 3).

The form of the curve relating enzyme formation induced by L-histidine with time (Fig. 6) is consistent with the idea that L-histidine, besides acting as inducer, was supplying respiratory energy, carbon skeletons or low molecular weight nitrogenous precursor material for enzyme synthesis. The course of enzyme synthesis under conditions where D-histidine was the inducing substance was linear (Fig. 7) and is comparable to 'gratuitous' conditions of induction (though the present experiments were conducted with washed suspensions and not with exponentially growing cultures). Markovitz & Klein (1955) described a similar linear production of induced α-amylase in washed suspensions of *Pseudomonas saccharophila.* The effect of succinate on the rate and final level of enzyme activity suggests that, in unsupplemented washed suspensions, the supply of respiratory substrates or carbon skeletons for synthetic purposes may have limited enzyme formation. Addition of ammonium chloride was without effect, suggesting that synthesis of enzyme in 'normal' organisms was not limited by supply of available nitrogen (Figs. 6, 7), though depletion of nitrogenous reserves by nitrogen starvation led to a decrease in the total enzyme produced in organisms induced with D-histidine (Table 4).

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On Distribution of Filamentous-Cluster Organisms (Krassilnikoviae) in Sea and Ocean Depths

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SUMMARY

The investigations carried out make it possible to conclude that filamentous-cluster organisms (Krassilnikoviae) rather form accumulations-layers at certain depths than get diffusely distributed in the sea and ocean water column. Possibly these layers occur at the junctions of water masses of different densities. The filamentous-cluster organisms have been found in all geographic zones of the world ocean, from Polar latitudes down to the equator.

It was established (Kriss & Mitskevich, 1959) that peculiar filamentous-cluster organisms occurring in sea and ocean water could be found mature and in considerable quantities on immersed slides within 6 hr. after these had been submerged to various depths. Taking into account the absence of filamentous-cluster organisms on filters through which were passed 20, 30 and 50 ml. water samples taken from the same horizons where filamentous-cluster organisms had been found on the slides, it was concluded that the organisms had developed on the slides and had not attached themselves from the water as ready-formed filaments with heads. However, the formation rate of these organisms seemed surprisingly high and therefore the investigation was continued. In the open part of the Black Sea slides were

Table 1. *The rate of appearance of filamentous-cluster organisms on slides after the latter were immersed into the depths of the Black Sea*

Depth slide immersion	Duration of exposure of slides						
(m.)	2·75 hr.	2 hr.*	1 hr.*	30 min.*	15 min.	5 min.	5 min.
25	Single individuals	Single individuals	Single individuals	Single individuals	.	.	.
100	Interlace	Interlace	Interlace	Interlace	Interlace	Interlace	Interlace
200	Interlace	Interlace	Interlace	Interlace	.	.	.
300	Interlace	Interlace	Interlace	Interlace	.	.	.
500	Single individuals	.	Interlace	Interlace	.	.	.
750	Interlace	Interlace	Interlace	Interlace	.	.	.
1000	Interlace	Interlace	Interlace	Interlace	.	.	.

* This exposure is for the upper slide; for the other slides it is much longer. In case of simultaneous immersion of slides to various horizons of the sea-water column the time difference between upper-slide exposure and lower-slide exposure (at 1000 m.) is *c.* 1 hr.

Table 2. *Distribution of filamentous-cluster organisms in the central part of the Pacific Ocean and in the Atlantic Ocean*

Depth of slide immersion (m.)	Station no.						
	3777	3782	3812	3823	347	363	377
	Pacific Ocean, 1958 (co-ordinates of location)				Atlantic Ocean, 1959 (co-ordinates of location)		
	32° 48′ 3″ N. 174° 02′ W.	24° 03′ 2″ N. 173° 31′ W.	14° 49′ S. 172° 56′ W.	23° 26′ 5″ S. 174° 39′ 54″ W.	66° 00′ N. 30° 00′ W.	33° 30′ N. 30° 00′ W.	00° 05′ N. 30° 00′ W.
	Duration of exposure of slides (hr.)						
	6	6	12	6	10	40	40
5	20	.	500	.	0	.	.
10	2	30	1,900	0	.	.	.
15	800	.	.	.	.	.	.
25	50	0	2100	0	4,160	.	.
35	0	.	.	.	.	.	.
50	310	210	240	20	13,150	840	5,050
75	200	25	1,100	2,000	70	.	.
100	.	65	390	0	.	990	13
150	.	145	1,300	0	.	.	.
200	.	130	0	4	.	130	130
250	.	.	365	.	.	.	.
300	.	60	20	.	.	.	.
400	.	.	80	.	.	.	.
500	.	.	0	.	.	90	45
1,000	.	.	.	.	.	680	105

The figures are the total number (sometimes approximated to tens) of filaments with heads on a glass area 15 cm.².

immersed to depths of 25, 100, 200, 300, 500, 750 and 1000 m. and exposed from 0·5 to 2·75 hr. Table 1 shows that even by a short exposure (30–60 min.) not infrequently a dense accumulation of filamentous-cluster organisms was found. These short time periods seemed obviously insufficient for filaments with heads to form on the slides. It became clear from the following experiment that single individuals or accumulations and interlacings of filamentous-cluster organisms got mechanically attached to the immersed slides. Slides were immersed to a depth of 100 m. and promptly removed. Although the immersion and removal of the slides took only 5 min., filaments with heads, either singly or in the form of accumulations or interlaced arrangements, were found on them. At the same time it became evident that samples taken by a bathometer from various depths in the Black Sea did not show filamentous-cluster micro-organisms even in such volumes as 500 or 1000 ml. This was proved by passing these volumes of water through membrane ultrafilters and examining them by microscopy.

These data make it possible to assume that, as a rule, filamentous-cluster organisms form layers and accumulations at certain depths rather than being diffusely distributed in the water column of seas and oceans. It is quite sufficient for a slide to pass through a layer or accumulation of this kind for it to collect a considerable number of filamentous-cluster organisms which are not infrequently interlaced in the form of long strings visible to the naked eye.

The turning of the cable exercised a negative influence on the 'catch', particularly during quick immersion. This was proved by immersing slides (12 × 14 cm.) in a horizontal position, the slides being fixed to the cable with the aid of rubber holders. It was observed that during immersion and removal the slides turned from side to side. Obviously, this turning of the cable in the water breaks up the accumulations of filamentous-cluster organisms, judging by the relatively small quantity of these organisms found on the large slides as compared with small slides.

It would appear that in sea and ocean depths there are formed more than one layer of filamentous-cluster organisms, since fairly frequently (Kriss & Mitskevich, 1959, Table 1) their quantity found in deeper horizons was greater than that found in less deep horizons. Possibly these layers occur at the junctions of water masses of different densities. This might account for relatively high quantities of filamentous-cluster organisms at the upper and lower boundaries of the Atlantic Current in the region of the North Pole.

A wide distribution of filamentous-cluster organisms in the world oceans has been confirmed by investigations carried out in the central part of the Pacific Ocean (expedition on S.S. *Vitijaz*, 1958) and along 30° W. in the Atlantic Ocean (expedition on S.S. *Lomonosov*, 1959). By the methods described earlier, and in addition to the data already published (Kriss & Mitskevich, 1959) it was established that filamentous-cluster organisms occurred at various depths of the ocean water column in subtropic and tropic-equatorial areas (Table 2). Thus, their geographic area in the world oceans extends from Polar latitudes to the equator.

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Transduction of Streptomycin Resistance in *Proteus mirabilis*

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SUMMARY

Five of eleven different temperate Proteus phages are capable of intrastrain transduction of a streptomycin resistance marker. Interstrain transduction of this marker was achieved with three of the five phages. Nuclear segregation and phenomic lag may both be involved in the delay in phenotypic expression of streptomycin resistance. The one transducing phage investigated appears to possess a normal phage genome in that transductant colonies are lysogenic and show lysogenic conversion, at multiplicities of phage input of less than 0·02, and under conditions where secondary infection on the plates can be excluded. However, under these conditions a few transductant clones were encountered which were not lysogenic. This indicates that the transducing and lysogenizing functions of this phage may be divorced from each other. Ultraviolet irradiation has a differential effect on its transducing and plaque-forming abilities.

INTRODUCTION

Coetzee (1958) demonstrated that many sewage phages isolated by lysis of different strains of *Proteus mirabilis* are also lytic for *P. vulgaris* strains and vice versa. To show that *P. mirabilis* and *P. vulgaris* possess genetic homologies and are varieties of a single genus (*P. hauseri*), the existence of some form of genetic exchange between them was sought. Bacteriophage-mediated genetic exchange of biochemical markers was tried (Coetzee, unpublished). These experiments were unsuccessful, possibly because the phages used were too virulent. With the discovery of a series of temperate Proteus phages (Coetzee & Sacks, 1959) fresh attempts at transduction were made. The restricted host range of these phages has thwarted the realization of our original objective but we could report (Coetzee & Sacks, 1960*a*) two intrastrain transducing systems in *P. mirabilis*, employing streptomycin resistance as marker.

The object of this paper is to report three additional *Proteus mirabilis* transducing systems and the occurrence of interstrain transduction. Because all the *P. mirabilis* strains used have identical biochemical reactions, natural biochemical markers could not be used in the three interstrain systems available. We have not been able to isolate mutants with biochemical markers or auxotrophic strains, and have confined our efforts to the streptomycin resistance marker. Once the problem of phenotypic delay is overcome, resistance to 1000 μg. streptomycin/ml. is a very satisfactory genetic marker.

METHODS

Media. The composition of the MacConkey-type agar and the broth employed have been described (Coetzee & Sacks, 1960*b*). The MacConkey type soft agar differed from the MacConkey-type agar in that it contained 0·7 % (w/v) instead of 1·7 % (w/v) agar. In some experiments 2 % (w/v) sodium citrate was added to the media. The MacConkey-type streptomycin agar was made by adding the necessary amount of streptomycin sulphate to the molten agar at 45°. Deoxyribonuclease, obtained from Seravac Laboratories, Cape Town, was used at a concentration of 40 μg/ml.

Organisms and phages. A series of 11 different phages isolated from lysogenic strains of Proteus (Coetzee & Sacks, 1959) were employed. Phages 12/57, 49/F7, 50/F7 and 54/F7 were obtained by ultraviolet irradiation of Proteus strains 12, 49, 50 and 54 respectively. The indicator organism for the phage obtained from Proteus 12 was Proteus 57, and the indicator organism for phages from Proteus 49, 50 and 54 was Proteus F7. Phages 34/13 and 36/13 were present in the supernatant fluids of 10-day broth cultures of Proteus 34 and 36, respectively. Proteus 13 was the indicator organism for both these phages. Phages $\frac{6}{24}/6$, $\frac{12}{57}/57$, $\frac{14}{20}/14$, $\frac{14}{20}/20$ and $\frac{24}{25}/14$ were present in the supernatant fluids of 10-day broth cultures of 6 and 24, 12 and 57, 14 and 20, 14 and 20, 24 and 25, respectively. The indicator organisms for these phages were Proteus strains 6, 57, 14, 20 and 14, respectively. The phages fail to clear broth cultures of their indicator organisms (Coetzee & Sacks, unpublished). Phages 34/13 and 36/13 are related serologically as are phages $\frac{14}{20}/14$ and $\frac{24}{25}/14$. Phage 12/57 is not antigenically related to these phages (Coetzee & Sacks, unpublished).

Proteus strains were maintained on nutrient agar slopes at 4°. Proteus mutants resistant to streptomycin were selected by plating *c.* 10^{11} cells on MacConkey agar containing 1000 μg. streptomycin/ml. These mutants, which are resistant to 1000 μg. streptomycin/ml., are marked by *str-r* following the identifying number of the organism. Organisms 113, 193, 57 and 13 are biochemically typical and identical *Proteus mirabilis* strains. The A phases (Belyavin, 1951) of 113 and 57 (or 57 *str-r*) do not swarm into each other and thus show a positive Dienes phenomenon (Dienes, 1947). It is concluded that they differ antigenically (Krikler, 1953). Organisms 193 and 13 (or 13 *str-r*) can be distinguished by the fact that they show different lytic patterns to a series of Proteus typing phages (Coetzee & Sacks, unpublished).

Preparation of phage lysates. Phage lysates with plaque-forming titres of *c.* 10^{10}/ml. were made by the modification (Adams, 1956) of the method of Hershey, Kalmanson & Bronfenbrenner (1943). Phages were propagated on the indicator organism or on its streptomycin resistant mutant. The latter phages are identified by the letters *str-r* appearing after the number of the indicator strain. Phage lysates were sterilized by mixing with 0·1 vol. of chloroform. Other phage techniques used have been described by Adams (1956). Irradiation of phage lysates was by a 30 W. Hanovia sterilamp which delivers more than 80 % of its output at a wave-length of 2537 Å. Lysates were irradiated in broth in thin layers in Petri dishes at a vertical distance of 50 cm. from the lamp (10 ergs/mm.^{2}/sec.). The contents of the Petri dishes were continuously agitated. During irradiation experiments the intensity of visible light was kept sufficiently low to avoid photoreactivation.

Transduction techniques. Phage active on the wild-type was grown successively on the corresponding streptomycin resistant mutant at least three times before transduction experiments were attempted. Transduction experiments were done as follows: three tubes, each containing 0·5 ml. of an aerated overnight culture of the potential recipient (titre 2×10^9/ml.) were centrifuged to clarity and the supernatant decanted. One ml. of the phage lysate of the streptomycin resistant strain (titre *c.* 5×10^9/ml.) was then mixed with the deposit in one of the tubes. One ml. of phage from the wild type donor (streptomycin sensitive indicator strain) of about the same titre was mixed with the deposit in the second tube and 1 ml. of broth was added to the deposit in the third tube. A fourth tube containing 1 ml. of phage suspension derived from the streptomycin resistant mutant served as the phage sterility control. After 15 min. adsorption at 37°, 0·1 ml. of the infected bacteria and the controls was spread to dryness on MacConkey agar plates. These plates were kept at 37° for 3 hr., and were then carefully overlayed with 5 ml. of MacConkey soft agar at 45° containing streptomycin sulphate to give a concentration of *c.* 1000 μg. streptomycin/ml. in the plates. After incubation for a further 24 hr. at 37°, the number of colonies appearing was recorded. Reconstruction experiments with known numbers of streptomycin resistant cells have shown that the 3 hr. old microcolonies are not disturbed by overlaying with the soft streptomycin agar. Lysates yielding positive results by the above test were mixed with either varying amounts of phage antiserum or deoxyribonuclease at 37° for 1 hr. The effect of these procedures was noted on the plaque-forming titre of the lysate and on the number of transductants produced.

Bacterial colony counts on MacConkey agar on samples from the adsorption tube measured bacterial survival.

Adsorption experiments of phage on bacteria were done as previously described (Coetzee, 1960).

Association of transduction and lysogenization. To investigate the association between transduction and lysogenization the technique of the transduction experiments was modified. One ml. of each of a series of doubling dilutions of phage from streptomycin resistant donors was added (in duplicate) to the usual constant number of recipient cells. The phage from the streptomycin sensitive donor at a multiplicity of input of 10, contained in 0·5 ml. was added to each of the one series of tubes and 0·5 ml. broth to each of the other series. In these experiments the possibility of lysogenization of the transductants by phage liberated on the plates from the background of phage-infected cells must be considered. With phage 34/13 *str-r* this possibility can be excluded by taking advantage of the fact that like phage P1 (Adams & Luria, 1958) it is dependent on Ca^{++} for adsorption (Coetzee & Sacks, unpublished). The infected cells were centrifuged after the adsorption period and resuspended in 1·0 ml. of broth containing 2 % sodium citrate. This suspension was plated on to MacConkey agar plates, containing 2 % sodium citrate, in the presence of potent phage antiserum. After 3 hr. incubation the plates were overlayed with MacConkey soft agar containing 2 % sodium citrate and streptomycin. These procedures remove unadsorbed phage, neutralize phage liberated on the plates, and prevent adsorption of phage not inactivated. Transductant colonies were picked off the plates and suspended in 1·0 ml. of broth containing 2 % sodium citrate. These suspensions were immediately plated, with phage antiserum, on to

MacConkey agar plates containing 2 % sodium citrate, and were also inoculated into citrate broth. The presence of liberated phage in these broth cultures after overnight incubation was determined by the production of plaques on Proteus 13. Single colonies from the last-mentioned citrate MacConkey agar plates were inoculated into plain broth, incubated overnight and titrated on Proteus 13 and streaked on MacConkey agar containing 0·001 N-Ca^{++}, and spotted at four sites with phage 34/13 *str-r*. The efficiency of the method used to eliminate secondary infection occurring on the plates was tested in reconstruction experiments. Proteus 13 was mixed with the non-transducing phage (34/13) at multiplicities of input ranging from 10 to 0·001. Non-adsorbed phage was removed by centrifuging, and the deposit was resuspended in broth containing sodium citrate. 0·1 ml. was plated with phage antiserum on to MacConkey agar plates containing sodium citrate. Suspensions of phage susceptible streptomycin resistant mutants diluted to yield *c.* 50 colonies per plate were then spread on all the plates. After 3 hr. incubation the plates were overlayed with soft agar containing streptomycin and sodium citrate. Forty entire colonies from each plate were streaked on MacConkey agar and phage 34/13 spotted at four sites along the streak.

RESULTS

Results of tranduction experiments are summarized in Table 1. These indicate that five of eleven different phage lysates examined are capable of intrastrain transduction of the streptomycin resistance marker and three of interstrain transduction of the same marker. Unsuccessful results in Table 1 represent failures using as potential recipients the streptomycin sensitive parental strain as host. While not presented in the table, negative results were also obtained with the few other available strains to which the phages adsorb. Adsorption experiments of transducing phage to recipient organisms reveal that more than 95 % adsorption occurs in 15 min. Table 2 shows the results of a typical experiment with phage 12/57 *str-r*. In the numerous

Table 1. *Summary of results of transduction experiments*

	Recipient Proteus				
Phage	57	13	14	113	193
12/57 *str-r*	+	0	0	+	0
34/13 *str-r*	0	+	0	0	+
$\frac{14}{20}$/14 *str-r*	0	0	+	0	0
$\frac{24}{25}$/14 *str-r*	0	0	+	0	0
36/13 *str-r*	0	+	0	0	+
	F7	57	20	6	
49/F7 *str-r*	–	0	0	0	
54/F7 *str-r*	–	0	0	0	
$\frac{12}{57}$/57 *str-r*	0	–	0	0	
$\frac{14}{20}$/20 *str-r*	0	0	–	0	
50/F7 *str-r*	–	0	0	0	
$\frac{6}{24}$/6 *str-r*	0	0	0	–	

+ = transduction successful; – = transduction unsuccessful; 0 = transduction not done because phage does not adsorb.

experiments done with the various systems, platings from cells infected with phage grown on streptomycin sensitive donors never showed any colonies, while on only five occasions were single colonies (spontaneous mutants) ever observed in platings from uninfected recipient cells. The transducing ability of a lysate was not affected by prior treatment with deoxyribonuclease, and bacteriophage was implicated as the genetic vector by abolition of transductants in experiments using a phage lysate previously treated with antiphage serum. The phage lysate from streptomycin resistant cells never produced colonies when plated on MacConkey-type agar.

Table 2. *Results of transduction experiments with phage* 12/57 *str-r*

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Transduction rate/phage particle adsorbed
Phage 12/57 *str-r*	1·0 ml.	0	0	1·0 ml.	0	0	—
Phage 12/57	0	1·0 ml.	0	0	0	0	—
Broth	0	0	1·0 ml.	0	0	0	—
Phage 12/57 *str-r* + DNase	0	0	0	0	1·0 ml.	0	—
Phage 12/57 *str-r* + anti-phage serum	0	0	0	0	0	1·0 ml.	—
Recipient bacteria	1×10^9	1×10^9	1×10^9	0	1×10^9	1×10^9	—
No. of colonies (Recipient strain 57)	194	0	0	0	190	0	2×10^{-6}
No. of colonies (recipient strain 113)	20	0	0	0	21	0	$2{\cdot}1\times10^{-7}$
Plaque-forming titre on strain 57	1×10^9/ml.	2×10^9/ml.	—	1×10^9/ml.	1×10^9/ml.	4×10^3/ml.	—

Tube 5 contained 1·0 ml. phage 12/57 *str-r* previously incubated with 40 μg. DNase at 37° for 1 hr.

Tube 6 contained 1·0 ml. phage 12/57 *str-r* previously incubated with anti-phage serum at 37° for 1 hr.

Plaque-forming titres determined just prior to mixing phage and recipient.

No. of colonies refers to the no. arising on MacConkey-type agar plates containing 1000 μg./ml. streptomycin, after plating 0·1 ml.

Transduction rates were calculated on the basis of the demonstrated 95 % adsorption.

Table 3. *Results obtained in typical experiments with transducing phages other than* 12/57 *str-r*

Phage	Recipient strain	Plaque-forming titre/ml.	No. of colonies on MacConkey agar containing 1000 μg./ml. streptomycin	Transduction rate/ phage particle adsorbed
34/13 *str-r*	13	1×10^9	10	$1{\cdot}1\times10^{-7}$
34/13 *str-r*	193	1×10^9	532	$5{\cdot}6\times10^{-6}$
36/13 *str-r*	13	6×10^9	33	$5{\cdot}8\times10^{-8}$
36/13 *str-r*	193	6×10^9	102	$1{\cdot}8\times10^{-7}$
$\frac{14}{20}$/14 *str-r*	14	5×10^{10}	50	$1{\cdot}1\times10^{-8}$
$\frac{24}{25}$/14 *str-r*	14	5×10^{10}	75	$1{\cdot}6\times10^{-8}$

1 ml. of phage suspension mixed with a deposit of 1×10^9 recipient bacteria. 0·1 ml. from adsorption tubes plated. Transduction rates calculated on the basis of the demonstrated 95 % adsorption.

Results obtained with phages other than 12/57 *str-r* are summarized in Table 3.

Intra- and interstrain transduction rates per phage particle adsorbed cannot be compared. This is due to differences in the lytic response of the recipient bacteria concerned.

The effect of ultraviolet irradiation on the transducing and plaque-forming ability of phage lysates 34/13 *str-r* is illustrated in Fig. 1. This reveals that the transducing ability of phage 34/13 *str-r* was more resistant to ultraviolet irradiation than its lytic activity. Phage 12/57 *str-r* (the only other phage investigated) has similar properties. No explanation for the initial rise in the transduction curve can be given. Similar findings have been recorded for other transducing systems (Garen & Zinder, 1955; Holloway & Monk, 1959; Loutit, 1959).

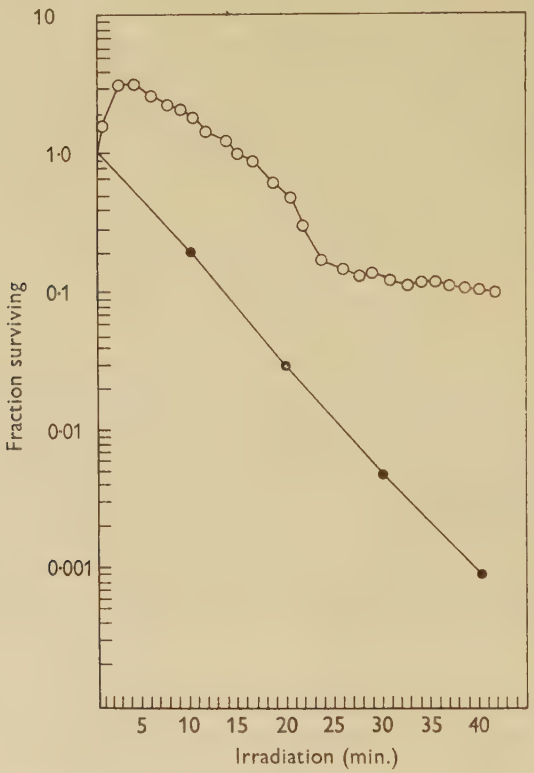

Fig. 1. Effect of ultraviolet irradiation on plaque-forming and transducing ability of phage 34/13 *str-r*. A lysate of phage 34/13 *str-r* (plaque-forming titre 1 × 10^9/ml.) was irradated in broth at 10 ergs/mm.^{2}/sec. At intervals, aliquots were removed and used in transduction experiments with Proteus 13 as recipient. The plaque-forming ability of the aliquots was also determined. Experiments were conducted in the dark. —○—, Transducing activity; —●—, plaque-formation.

The phenomenon of delay in expression of transduced streptomycin resistance was investigated by plating 0·1 ml. of the transducing mixture and the controls at intervals directly on to MacConkey agar plates containing different concentrations of streptomycin. Results are presented in Table 4. Because of lysis of some recipient bacteria and because infection may not be simultaneous this method does not allow the accurate determination of the number of divisions required for expression of resistance to 1000 μg. streptomycin/ml. The results show, however, that resistance can be expressed after a doubling of the population surviving after 15 min. More transductants are recovered earlier on low concentrations of streptomycin indicating that complete phenotypic expression of the transduced character takes place gradually. Similar results were encountered by Watanabe & Watanabe (1959) for streptomycin indifference transduction in *Salmonella typhimurium* and by Alexander & Leidy (1953) for streptomycin resistance transformation in *Haemophilus influenzae*. Cells of *Proteus mirabilis* contain from 1 to 4 nuclei (Coetzee & Sacks, 1960*c*). Streptomycin resistance is recessive to sensitivity in *Escherichia coli* (Lederberg, 1951). If this also applies to Proteus, the time required for segregation

Table 4. *Results of a transduction experiment with phage* 12/57 *str-r and recipient strain* 57 *to demonstrate the properties of phenotypic delay in the expression of streptomycin resistance*

Min. after mixing phage and recipient at 37°	Viable bacteria per ml.	No. of colonies on MacConkey agar containing streptomycin (μg./ml.)				
		125	250	500	1000	2000
15	$3{\cdot}1\times10^{6}$	0	0	0	0	0
60	$3{\cdot}5\times10^{6}$	15	9	2	0	0
90	$6{\cdot}5\times10^{6}$	28	17	8	2	0
120	$9{\cdot}0\times10^{6}$	54	36	34	26	9
150	$1{\cdot}3\times10^{7}$	198	165	143	112	61
180	$2{\cdot}3\times10^{7}$	308	217	181	170	121
240	$6{\cdot}0\times10^{7}$	621	631	497	516	412
300	$1{\cdot}5\times10^{8}$	932	912	820	844	722
360	$2{\cdot}4\times10^{8}$	1192	1020	932	964	934

A deposit of 1×10^{7} bacteria was infected with 1·0 ml. of phage 12/57 *str-r* at a multiplicity of input of 5. Six identical adsorption tubes were pooled to provide sufficient transducing mixture for inoculation of plates with 0·1 ml.

Table 5. *Results of a reconstruction experiment to test for lysogenization of colonies by phage liberated on transduction plates*

1·0 ml. of appropriate dilutions of phage 34/13 added to deposits of *c.* 1×10^{9} Proteus 13. After adsorption for 15 min. tubes were centrifuged and deposits resuspended in 1·0 ml. citrate broth. 0·1 ml. plated, with phage antiserum, on citrate MacConkey agar platet. *c.* 50 cells of Proteus 13 *str-r* inoculated on to each plate. After 3 hr. incubation plates overlayed with agar containing citrate and streptomycin.

M.I.	No. of colonies tested	No. of phage susceptible colonies
10	40	40
1	40	40
0·1	40	40
0·01	40	40
0·001	40	40

Colonies present after 18 hr.-incubation at 37° tested for phage susceptibility, by picking off entire colony, streaking on plain MacConkey plates and spotting with phage 34/13. M.I. = multiplicity of phage input.

of a homonucleate cell (Newcombe & Hawirko, 1949) may be one factor involved in the delay in phenotypic expression. The phenotypic expression of a different gene may have to await the exhaustion of pre-existing material in a cell; that this phenomic delay (Lamanna & Mallette, 1953) may be an additional factor involved is indicated by the fact that the transition from partial to complete resistance is lengthy and gradual.

All transductant clones are perfectly stable for their newly acquired character and although the morphological phase B (Belyavin, 1951) is commonest, all other phases, except the R variants (Coetzeé, 1959), are seen.

Table 6. *Results of an experiment with phage* 34/13 str-r *to show the relationship between multiplicity of infection, lysogenization and transduction*

Deposit from adsorption tubes resuspended in broth containing 2 % sodium citrate. 0·2 ml. plated together with 0·2 ml. anti-phage serum on to MacConkey agar containing 2 % sodium citrate. After 3 hr. incubation, plates overlayed with agar containing citrate and streptomycin. Colonies present after 18 hr. incubation at 37° tested for spontaneous liberation of phage and phage susceptibility. Denominators indicate no. of clones tested. M.I. = Multiplicity of phage input.

1·0 ml. of doubling dilutions of transducing phage (plaque-forming titre 1×10^{10}/ml.) added to deposits of 1×10^{9} of strain 13 and mixed with

0·5 ml. wild phage (titre 2×10^{10}/ml.)		0·5 ml. broth		
			Transductant clones	
M.I. 10 plus	Total no. transluctant clones	M.I.	Total no.	No. lysogenic and phage insusceptible
10	> 1500	10	> 1500	30/30
5	> 1500	5	> 1500	30/30
2·5	> 1500	2·5	> 1500	30/30
1·3	> 1500	1·3	> 1500	30/30
0·6	982	0·6	1040	30/30
0·3	520	0·3	542	30/30
0·2	176	0·2	189	30/30
0·1	128	0·1	114	30/30
0·04	71	0·04	64	30/30
0·02	41	0·02	36	18/20*
0·01	18	0·01	20	15/15
0·005	13	0·005	13	8/10*
0·002	4	0·002	6	5/6*
0·001	1	0·001	3	3/3
0·0005	0	0·0005	0	—
0·0002	0	0·0002	0	—
0·0001	0	0·0001	0	—
0·00005	0	0·00005	3	3/3

*These 5 non-lysogenic clones were phage susceptible.

No abortive transductants (Stocker, 1956) were encountered. This may be due to the possibility that streptomycin resistance is recessive to sensitivity and also to the fact that there is a delay in phenotypic expression of streptomycin resistance. Watanabe & Watanabe (1959) advanced similar reasons for their failure to observe abortive transductants in the transduction of streptomycin resistance in the *Salmonella typhimurium* system.

Results of experiments to demonstrate the relationship between lysogenization and transduction are presented in Tables 5 and 6. In the reconstruction experiment (Table 5), entire colonies were streaked on the plates prior to multiple spotting with phage. It is unlikely that these results can all be ascribed to the presence of non-lysogenic segregants. It is concluded that under conditions prevailing in these experiments secondary infection resulting in lysogenization of colonies arising on the plates does not occur. Results in Table 6 indicate that the yield of transductants is roughly proportional to phage input and is insensitive to assistance by non-transducing phage; and that the vast majority of transductant clones, even at low multiplicities of input, are lysogenic and liberate phage spontaneously. No discrepancies were noted between the two broth cultures tested for each clone. The lysogenic transductant clones are not only phage insusceptible but also fail to adsorb homologous phage. This phenomenon, which is due to lysogenic conversion (Coetzee, 1960), has precluded the use of these transductants as donors for further experiments and has also prevented the use of lysogenized cells as recipients. The rare non-lysogenic transductants discovered were sensitive to homologous phage. No defective lysogenic transductant colonies were encountered.

Transduction experiments attempted with phage liberated spontaneously by the lysogenic transductant clones (plaque-forming titre 1×10^6/ml.) were unsuccessful.

DISCUSSION

In the intrastrain transduction previously described (Coetzee & Sacks, 1960*a*), donor and recipient cells were isogenic except for the marker in question. The inter-strain transduction achieved here shows that the transferred genetic fragment is also capable of integration in a different genome. The restricted host range of the phages has prevented any inter-genus hybridization of the kind achieved by Lennox (1955) with phage P1.

Five of eleven different Proteus phages are capable of transducing the *str-r* marker. This indicates a position similar to that in Salmonella where many temperate phages can transduce (Edwards, Davis & Cherry, 1955). A different situation exists in *Escherichia coli* where only two phages (which are also related serologically) are competent in general transduction (Jacob, 1955).

The transduction of only one marker has been investigated in the various Proteus systems described. The title of general or common transduction as exemplified by Zinder & Lederberg's (1952) original system using *Salmonella typhimurium* and phage P22 and later extended to *Escherichia coli* by the use of phage P1 (Lennox, 1955; Jacob, 1955) can therefore not be applied to these processes at this stage. The rates of transduction per phage particle are of the same order as those found in general transduction. However, unlike transducing phages P1, and possibly P22 (Starlinger, 1958; Stocker, 1959) transducing phage 34/13 *str-r* appears to possess a complete phage genome, since at multiplicities of phage input of 0·02 and less, most transductant clones are lysogenic and show conversion. The rare exceptions encountered indicate that the lysogenizing and transducing properties of this phage are functions which may be divorced. Separation of these activities of phage P22 has been reported under a variety of conditions (Hartman, 1957). The differential effect which ultraviolet irradiation of phage 34/13 *str-r* has on its plaque-forming and transducing abilities affords a further example of how phage activities may be divorced from each other. While the failure to transduce the streptomycin resistance marker with phage liberated spontaneously by the lysogenic transductants is probably due to the low multiplicity of phage input, it also indicates that the high-frequency tranduction system described by Morse, Lederberg & Lederberg (1956*a*, *b*) for the λ-Gal phage system in *E. coli* is not being encountered. The λ-Gal phage system also differs from the Proteus transduction mechanism described here in that only induced lysogenic *E. coli* yield active lysates. Further differentiation from the λ system may be made when more genetic markers are discovered in Proteus.

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The Growth of Micro-organisms in Relation to their Energy Supply

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SUMMARY

When *Streptococcus faecalis* was grown anaerobically in a complex medium containing D-glucose, D-ribose or L-arginine as energy source the dry wt. of organism produced was proportional to the concentration of the energy source in the medium. However, *S. faecalis* will not grow in a defined medium with arginine as the energy source unless glucose is present at the same time. The anaerobic growth of both *Saccharomyces cerevisiae* and *Pseudomonas lindneri* was proportional to the concentration of glucose in the medium and the yield coefficient—defined as g. dry wt. organism/mole glucose—of the former was the same as that of *S. faecalis* grown upon glucose and approximately twice that of *P. lindneri*. Calculation of the g. dry wt. organism/mole adenosine triphosphate synthesized for these three organisms gave values ranging from 12·6 to 8·3 with an average of 10·5. These results suggest that, under anaerobic conditions, the yield of *S. faecalis*, *S. cerevisiae* and *P. lindneri* was proportional to the amount of ATP synthesized. When *Propionibacterium pentosaceum* was grown anaerobically with glucose, glycerol or DL-lactate as energy source there was, in all three cases, a linear relationship between the dry wt. of organisms produced and the concentration of the energy source in the medium. The values of the yield coefficients obtained were compatible with the formation of approximately 4 mole ATP/mole glucose, 2 mole ATP/mole glycerol and 1 mole ATP/mole lactate.

INTRODUCTION

Over the past twenty years an enormous literature has grown up around the quantitative aspects of microbial nutrition. The majority of the papers published have been concerned with the relationship between the growth of an organism, usually estimated indirectly either by optical methods or by the titration of an acidic end-product, and the amount of some essential nutrient, other than the energy source, in the medium. The fact that there is a linear or almost linear relationship between growth and the amount of a particular nutrient has led to the development of highly sensitive specific methods for the quantitative estimation of amino acids, purines, pyrimidines and the B group of vitamins. In contrast there has been very little work on the relationship between growth and the amount of the energy source in the medium. The most complete study of recent years was that of Monod (1942) who studied the growth of three organisms—*Bacillus subtilis*, *Escherichia*

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coli and *Salmonella typhimurium*. These organisms were grown anaerobically on a mineral medium with a wide range of carbohydrates as energy source, and as long as the energy source was the factor limiting growth, the dry weight of organism produced was proportional to the weight of energy source added. It is of particular interest that for a given carbohydrate the three organisms produced approximately the same dry wt. yields.

DeMoss, Bard & Gunsalus (1951) studied the growth of *Streptococcus faecalis* and *Leuconostoc mesenteroides* and expressed their results as dry wt. organism/mole energy source provided. Both of these organisms have complex nutritional requirements and both obtain their energy for growth by the anaerobic catabolism of carbohydrates. Thus, *S. faecalis* converts glucose to lactic acid and *L. mesenteroides* to an equimolecular mixture of lactic acid, ethanol and carbon dioxide. The yields of the fermentation products were almost quantitative, suggesting that very little of the glucose was assimilated and that it was used almost exclusively as an energy source. This is in marked contrast to the aerobes studied by Monod (1942) which, since they were grown upon a simple mineral medium, used a considerable portion of the energy source for the synthesis of cell material. DeMoss *et al.* (1951) (see also Sokatch & Gunsalus, 1957) found that with both *S. faecalis* and *L. mesenteroides* there was a linear relationship between the dry weight of organism produced and the amount of glucose added, and that with glucose as the energy source the yield of *S. faecalis* was significantly greater than that of *L. mesenteroides*. They concluded from these results that, because both organisms used glucose mainly as an energy source, and because growth was proportional to the amount of energy supplied, *S. faecalis* obtained more energy/mole glucose than did *L. mesenteroides* and that the latter organism fermented glucose by a mechanism other than the Embden–Meyerhof pathway. This is a conclusion of considerable importance. The work of Gunsalus & Gibbs (1952) and more recently of Hurwitz (1958) has shown that, not only does *L. mesenteroides* ferment glucose by a new pathway involving xylulose-5-phosphate (Heath, Hurwitz, Horecker & Ginsberg, 1958) as the key intermediate, but also that the process yields only 1 mole of adenosine triphosphate (ATP)/mole of glucose, as compared with the 2 mole of ATP/mole of glucose produced by the Embden–Meyerhof pathway which is the fermentation mechanism of *S. faecalis*. From this it would appear that the growth of *S. faecalis* and *L. mesenteroides* is related to the amount of ATP produced.

In order to study further the relationship between the growth of *Streptococcus faecalis* and the energy supply we have measured the weight of organism produced when it was grown on D-glucose, D-ribose and L-arginine. Arginine was studied because it was observed that addition of this compound to the medium increased the yield of organism. The work of Akamatsu & Sekine (1951) established that *S. faecalis* converts arginine quantitatively to ornithine with citrulline as the intermediate and Knivett (1954*a*) subsequently found that the breakdown of citrulline was coupled with the synthesis of ATP. Thus, in terms of mole ATP formed/mole of substrate, arginine yields half as much ATP as glucose. In addition to these experiments the growth of *Saccharomyces cerevisiae* and *Pseudomonas lindneri* on glucose was measured; these two organisms were selected because both ferment glucose to ethanol and carbon dioxide. The former uses the Embden–Meyerhof pathway which gives 2 mole ATP/mole glucose and the latter the Entner–Doudoroff pathway (Gibbs & DeMoss, 1954) which gives one mole ATP/mole glucose (see Elsden & Peel, 1958). Finally, the growth of *Propionibacterium pentosaceum* was measured on glucose, glycerol and DL-lactate. A preliminary account of this work has been published (Bauchop, 1958).

METHODS

Organisms. The following cultures were used: *Streptococcus faecalis* (NCTC 6783) a laboratory strain of *Saccharomyces cerevisiae*; *Propionibacterium pentosaceum* strain E.2.1. from the collection of Dr C. B. van Niel; *Pseudomonas lindneri* ATCC No. 10988, NCIB No. 8938.

Media

Stock salt solution (*S.S.S.*) MgO, 10·75 g.; $CaCO_3$, 2·0 g.; $FeSO_4.7H_2O$, 4·5 g.; $ZnSO_4.7H_2O$, 1·44 g.; $MnSO_4.4H_2O$, 1·12 g.; $CuSO_4.5H_2O$, 0·25 g.; $CoSO_4.7H_2O$, 0·28 g.; H_3BO_3, 0·06 g.; HCl (concn.), 51·3 ml. This mixture was made up in distilled water to 1 l.

Streptococcus faecalis *partially defined medium.* 10 g. casein hydrolysate (Oxoid) were dissolved in 100 ml. distilled water, 15 ml. N-NaOH added and the mixture allowed to stand for 15 min. The precipitate which formed was filtered off and 15 ml. N-H_2SO_4 added to the filtrate. The following mixture was then added to this solution: KH_2PO_4, 7·5 g.; K_2HPO_4, 7·5 g.; $(NH_4)_2SO_4$, 2 g.; adenine, 20 mg.; DL-tryptophan, 10 mg.; S.S.S., 5 ml.; asparagine, 1 g.; L-cysteine, 0·1 g.; nicotinamide, 1 mg.; Ca pantothenate, 1 mg.; riboflavin, 1 mg.; pyridoxin, 2 mg.; folic acid, 0·1 mg.; biotin, 0·1 mg.; adjusted to pH 6·6–6·8 and the volume made up to 1 l. with distilled water.

Saccharomyces cerevisiae *medium.* This medium was a modification of that used by White & Munns (1951): casein hydrolysate (Oxoid), 10 g.; Na_2HPO_4, 2 g.; S.S.S., 5 ml.; thiamine HCl, 0·2 mg.; riboflavin, 0·1 mg.; nicotinamide, 5·0 mg.; *p*-aminobenzoic acid, 0·3 mg.; pyridoxin, 1·0 mg.; Ca pantothenate, 0·5 mg.; *meso*-inositol, 50 mg.; biotin, 6 μg.; adjusted to pH 5·0 and made up to 1 l. with distilled water. For anaerobic growth this medium was supplemented with ergosterol (Andreasen & Stier, 1953): Ergosterol (63·8 mg.) and Tween 80 (6·5 ml.) were boiled with absolute ethanol until the ergosterol was dissolved, and the solution made up to 25 ml. with absolute ethanol; 3 ml. of this solution were added to 150 ml. of the medium at the time of the inoculation.

Propionibacterium pentosaceum *medium.* This was a modified version of the defined medium of Delwiche (1950). 5 g. Casein hydrolysate (Oxoid) were dissolved in 100 ml. distilled water, 15 ml. N-NaOH added and the mixture allowed to stand for 15 min. The precipitate which formed was filtered off and 15 ml. N-H_2SO_4 added to the filtrate. The following additions were then made: $CH_3COONa.3H_2O$, 8 g.; K_2HPO_4, 7·5 g.; KH_2PO_4, 7·5 g.; S.S.S., 5 ml.; L-cysteine, 50 mg.; sodium thioglycollate, 0·2 g.; DL-tryptophan, 0·1 g.; adenine, 10 mg.; guanine, 10 mg.; uracil, 10 mg.; xanthine, 10 mg.; Ca pantothenate, 1 mg.; thiamine HCl, 1 mg.; biotin, 0·5 mg.; *p*-aminobenzoic acid, 1 mg.; riboflavin, 4 mg.; adjusted to pH 6·6–6·8 and made up to 1 l. with distilled water.

Pseudomonas lindneri *medium.* This consisted of 1 % (w/v) Bacto Peptone and 1 % (w/v) Difco yeast extract.

Cultivation and harvesting of organisms

Direct dry weight measurements. Conical flasks (250 ml.) were prepared containing 150 ml. medium and a test-tube containing 3 ml. of 15% (w/v) pyrogallol was placed in each flask. The flasks were sterilized by autoclaving at 15 lb. sq.in. for 15 min. The required amounts of glucose and inoculum were then added, and in the case of the yeast, the appropriate amount of ergosterol solution. Finally, 3 ml. of 10% Na_2CO_3 were added to the test-tube containing pyrogallol. The flasks were closed with rubber stoppers, sealed with paraffin wax, and incubated at the appropriate temperature. *Streptococcus faecalis* was grown at 37°; *Saccharomyces cerevisiae*, *Propionibacterium pentosaceum* and *Pseudomonas lindneri* were grown at 30°. When growth had reached completion the organisms were harvested by centrifugation, washed three times with 100 ml. distilled water, washed into beakers and dried at 100° to constant weight.

Dry weight by turbidity measurements. Organisms were grown in $6 \times \frac{3}{4}$ in. test-tubes containing 9 ml. medium. Substrates, inocula and other additions, where applicable, were made to a final vol. of 10 ml. A wad of absorbent cotton wool was then pushed down over the sterile test-tube plug and on this were placed 5 drops each of 15% (w/v) pyrogallol and 10% (w/v) Na_2CO_3, in that order. The tube was sealed with a rubber stopper and incubated at the appropriate temperature. Growth was measured turbidimetrically in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex) until it reached completion, when the optical density was read in a Spekker Absorptiometer, with an H 508 filter. The Spekker readings were converted into dry weights by reference to a standard curve relating optical density to dry weight.

Continuous culture apparatus. The apparatus used was that described by Rosenberger & Elsden (1960). Anaerobic conditions were obtained by aerating the culture vessel with oxygen-free nitrogen.

Analytical methods

Radioactivity measurements. Radioactivity was measured with an end-window Geiger–Muller counter. A sample of the uniformly labelled D-(^{14}C)-glucose (obtained from the Radiochemical Centre, Amersham, Buckinghamshire) solution was diluted with an equal volume of absolute ethanol, and counted directly by spreading 25 μl. of this solution containing 125 μg. glucose on brass disks (Ormerod, 1956) and drying under an infra-red lamp. Results, corrected for background, are given as counts/min. at infinite thinness.

Glycerol was estimated by the method of Burton (1957) with glucose as a standard (O'Dea & Gibbons, 1953).

Lactate was estimated by the method of Elsden & Gibson (1954).

Arginine dihydrolase was assayed by the method of Knivett (1954*b*).

Definition of terms used. To facilitate comparison of the results obtained with the various substrates and organisms we express our results as: g. dry wt. organism produced/mole substrate added. We call this ratio the yield coefficient, Y, with the substrate given as a subscript; thus Y_{glucose} refers to the yield coefficient of an organism growing upon glucose as the energy source. The yield coefficient has, in the past, been given a variety of names, for example, the yield constant, the growth yield constant, the molar growth yield. Our reasons for introducing a further term for this ratio are as follows. Herbert (1958) has shown that the value of Y decreases with decreasing growth rate. Since the growth rate is a function of the substrate concentration (Monod, 1942) it is clear that, in batch cultures, once this critical substrate concentration is reached, the value of Y will begin to decrease. However, the concentration of the substrate at which the growth rate begins to be affected is very low as compared to the initial substrate concentration (Monod (1942)), and in consequence the value of Y as defined above is not significantly affected. On the other hand, it seems to us inappropriate to call a ratio a constant when it is manifestly not constant under all conditions.

The ATP yield of a metabolic process is defined as: mole ATP produced/mole substrate used. Where both the yield coefficient Y and the ATP yield of the metabolic process involved are known it is possible to calculate the dry wt. of organism produced/mole ATP formed; the ratio so obtained we call Y^{ATP}.

RESULTS

Yield coefficients

All experiments were carried out under strict anaerobiosis and the media were so constituted that the energy source was the factor which limited growth. To ensure that this was the case the yields were measured over a range of substrate concentrations. Figure 1 shows plots of the growth yields of *Streptococcus faecalis* on the complex medium against the concentration of the substrates D-glucose, D-ribose and L-arginine, respectively. It will be noted from Figs. 1 and 2 that although there was a linear relationship between the wt. of *S. faecalis* produced and the concentrations of the three substrates, the curves did not pass through the origins. Rather, at zero substrate concentration, they intersected the ordinate at a positive value for dry wt. This could mean either, that over a range of substrate concentrations less than those tested, the slopes of the curves and hence the yield coefficients were greater than at higher substrate concentrations. Or, that there was present in the media a small but significant amount of an unknown energy source upon which the organism grew. We think that the latter is the correct explanation for there was always some growth in both the media used in the absence of an added energy source. The magnitude of this 'blank' growth was too small to measure accurately.

Figure 2 shows curves relating growth yields of *Streptococcus faecalis* and *Saccharomyces cerevisiae* to glucose concentration. In the case of *S. cerevisiae* it was found that the organism would grow in the absence of oxygen only when the medium was reinforced with ergosterol (cf. Andreasen & Stier, 1953); the steroid was not required for aerobic growth. The slopes of the two curves shown in Fig. 2 are identical, implying that the values of Y_{glucose} for *S. faecalis* and *S. cerevisiae* under these conditions are the same. It will be noted from Fig. 2 that even when the partially defined medium was used there was always some growth of *S. faecalis* in the absence of added carbohydrate. Figure 3 shows the relationship between the growth of *Pseudomonas lindneri* and the glucose content of the medium. The slope suggests that Y_{glucose} is about half that of *S. faecalis* and *S. cerevisiae*. In Fig. 4 are plotted the results of experiments with *Propionibacterium pentosaceum* grown anaerobically on glucose, glycerol and lactate. Comparing the slopes of these curves with those

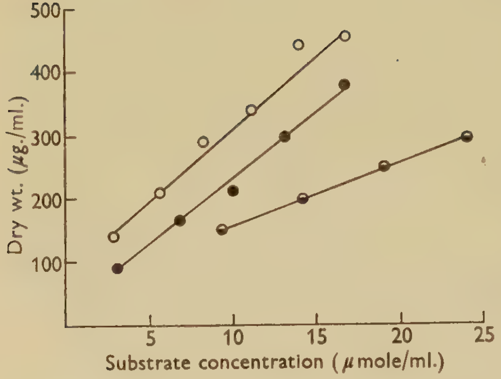

Fig. 1

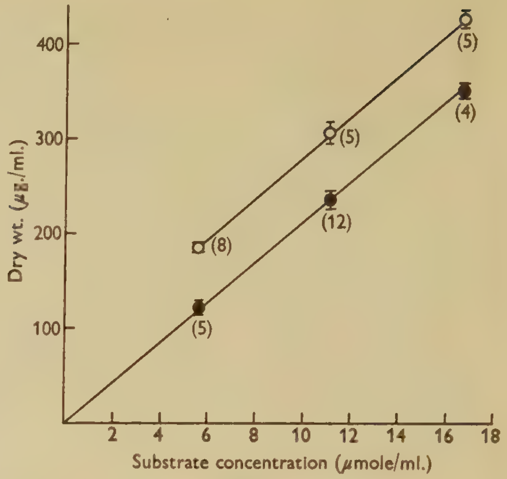

Fig. 2

Fig. 1. Growth of *Streptococcus faecalis* on glucose, ribose and arginine. Organism grown on complex medium with glucose, ribose and arginine as energy source. Incubation at 37°. Growth measured turbidimetrically and dry wt. read from a standard curve relating optical density to dry wt. ○—○ = growth on glucose; ●—● = growth on ribose; ◒—◒ = growth on arginine.

Fig. 2. Growth of *Streptococcus faecalis* and *Saccharomyces cerevisiae* on glucose. *S. faecalis* grown on partially defined medium and incubated at 37°; *S. cerevisiae* grown on medium given in text and incubated at 30°. At the end of growth cells harvested, washed and dried at 100° to constant weight. ○—○ = *S. faecalis*; ●—● = *S. cerevisiae*. Each point is the mean of the number of estimations shown in parentheses; the standard deviations from the mean are represented by the heights of the vertical bars.

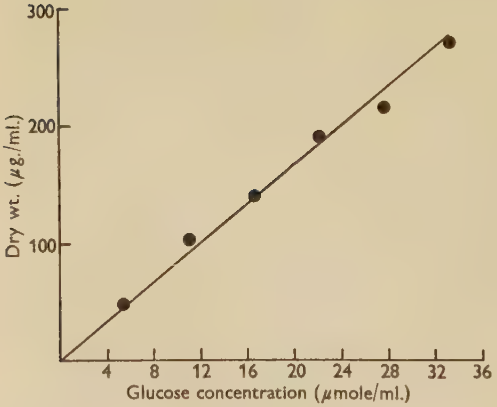

Fig. 3

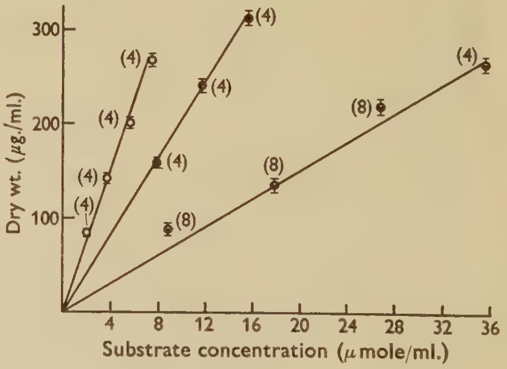

Fig. 4

Fig. 3. Growth of *Pseudomonas lindneri* on glucose. Incubation at 30°. Growth measured turbidimetrically and the dry wt. read from a standard curve relating optical density to dry wt.

Fig. 4. Growth of *Propionibacterium pentosaceum* on glucose, glycerol and lactate. Incubation at 30°. At the end of growth, organism was harvested, washed and dried at 100° to constant weight. Each point is the mean of the number of estimations shown in parentheses; the standard deviations from the mean are represented by the heights of the vertical bars. ○—○ = glucose; ●—● = glycerol; ◒—◒ = sodium lactate.

in Fig. 2 for *S. faecalis* and *S. cerevisiae* it will be seen that the value of Y_{glucose} of *P. pentosaceum* is considerably greater than that of the other two organisms when grown on the same substrate, suggesting that *P. pentosaceum* obtains more energy from glucose than either *S. faecalis* or *S. cerevisiae.*

The growth of Streptococcus faecalis *on* L*-arginine*

It has already been shown that when L-arginine was added to the complex medium the yield of *Streptococcus faecalis* was increased and that the growth was a linear function of the amount of arginine added. When, however, the experiment was repeated with the partially defined medium the addition of arginine did not increase the slight growth above that obtained with the medium alone; it will be recalled that there was good growth on this medium when glucose was the substrate (see Fig. 2). Two explanations appeared possible: either the complex medium contains a factor which is essential for growth on arginine; or the inoculum contained no arginine dihydrolase, the enzyme system which is concerned with the catabolism of arginine (Slade & Slamp, 1952). This latter hypothesis was found to be untenable because organisms known to contain arginine dihydrolase failed to grow when inoculated into the partially defined medium to which only arginine had been added. It was next found that addition of arginine to the partially defined medium containing a small amount of glucose raised the yield of organism above that obtained with the medium to which only glucose had been added; but the response to arginine was irregular. Figure 5 shows the results of an experiment in which four different glucose concentrations were used, and it will be seen that a linear response to arginine was obtained only with the highest glucose concentration. However, even with the highest glucose concentration tested the results were not reproducible. These experiments suggested that whilst glucose promoted the utilization of arginine for growth, the factor which was essential for growth under these conditions disappeared from the medium during growth at a rate which varied from experiment to experiment. Since glucose was known to be utilized under these conditions it seemed possible that it was the missing factor and that the irregular results observed were due to a utilization of all the glucose in the early stages of growth. Slade & Slamp (1952) had in fact found in their experiments that arginine only began to disappear from the medium when 70% of the glucose had been utilized, and the concentration of glucose in their medium was considerably greater than that used in these experiments. To test this hypothesis *S. faecalis* was grown in continuous culture on the partially defined medium in the apparatus described by Rosenberger & Elsden (1960).

With this technique fresh medium is added continuously to the culture at a constant rate, and the effect of supplementing with arginine could thus be investigated under conditions where glucose was present at all times albeit at a low concentration. The culture was started using the partially defined medium plus glucose at a concentration of 2·4 μmole/ml. When the concentration of cells approached the expected value, the flow of medium into the culture vessel was started and a steady state was reached 18 hr. after inoculation. The culture was allowed to grow in this way for a further 24 hr. during which time the cell concentration and the pH value of the culture were measured at suitable time intervals. Arginine was then added to the medium in the reservoir to give a final concentration of 9·5 μmole/ml.; almost immediately the cell concentration began to increase and 10 hr. after the addition of the arginine was started a new steady state was reached which was maintained for a further 30 hr. The complete protocol of the experiment is given in Table 1; Fig. 6 is a plot of the dry wt. organism/ml. against time from the eighteenth hour onward. The mean dry wt. organism/ml. during the glucose steady state was 119 μg. ml. and in the steady state in the presence of glucose + arginine 219 μg. ml. The yield of organism due to arginine was therefore 100 μg. ml. The concentration of arginine in the medium was 9·5 μmole/ml., giving a Y_{arginine} of 10·5; this agrees well with the value of 10 obtained in the experiment with the complex medium.

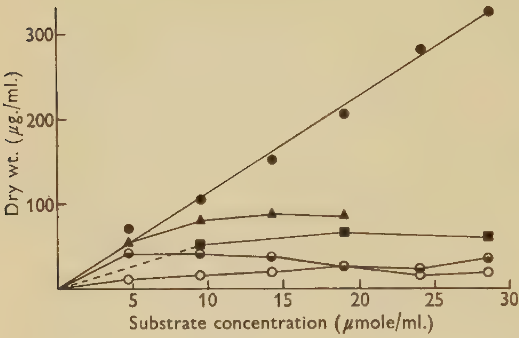

Fig. 5

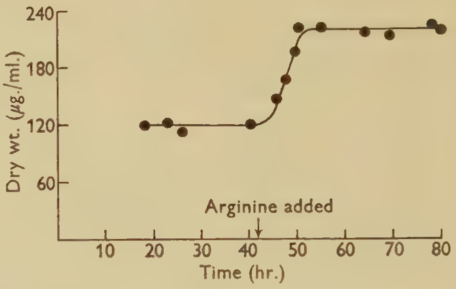

Fig. 6

Fig. 5. Effect of glucose on the growth of *Streptococcus faecalis* in partially defined medium containing arginine. Incubation at 37°. Growth measured turbidimetrically and the dry wt. read from a graph relating optical density to dry wt. ○—○ = no glucose; ◒—◒ = 0·56 μmole glucose/ml.; ■—■ = 2·78 μmole glucose/ml.; ▲—▲ = 4·17 μmole glucose/ml.; ●—● = 5·56 μmole glucose/ml.

Fig. 6. Growth of *Streptococcus faecalis* in continuous culture. The partially defined medium was used which contained initially 2·5 μmole glucose/ml. At 41·75 hr., marked by arrow, sterile arginine solution was added to the reservoir to give a final concentration of 9·5 μmole arginine/ml. Growth measured turbidimetrically and dry wt. read from a graph relating optical density to dry wt.

Assimilation of glucose by Streptococcus faecalis

In calculating the yield coefficients it was assumed that all the substrate added was catabolized. To test the validity of this assumption *Streptococcus faecalis* was grown on uniformly labelled D-(^{14}C)-glucose, using 150 ml. of the partially defined medium containing 0·1 % (w/v) D-(^{14}C)-glucose, specific activity = 838 counts/min./mg. When growth ceased the optical density of the culture was measured with the Spekker absorptiometer and the organisms harvested by centrifuging at 2° in an International refrigerated centrifuge. The organisms were washed once with 100 ml. 5 % (w/v) trichloroacetic acid (TCA), twice with 50 ml. 5 % TCA and once with 50 ml. 0·05 % (w/v) sodium lactate. This last was used as a carrier to remove traces of radioactive lactate. The precipitate so obtained was washed once with 50 ml. distilled water and suspended evenly in 100 ml. distilled water. Samples (5 ml.) were filtered on a circle of Whatman No. 40 filter paper, as described by Ormerod (1956) and the precipitate washed sequentially with 50 % (w/v) aqueous acetone and acetone and, after drying under the infra-red lamp the filter papers were prepared for counting. Since about 1·4 mg. cell residues were counted on disks of area 2·8 cm.^{2} self-absorption could be neglected. The elaborate washing procedure was used to ensure that the samples were free from non-cellular radioactive materials, particularly end products of the fermentation. To check the efficacy of the method organisms from a culture grown on glucose were suspended in the supernatant fluid obtained by centrifuging a culture grown on (^{14}C)-glucose. This

Table 1. *Growth of* Streptococcus faecalis *in continuous culture with glucose and arginine as energy sources*

Organism grown in partially defined medium containing, initially, 2·4 μmole glucose/ml. After 41·75 hr. arginine was added to the reservoir to give a final concentration of 9·5 μmole/ml. medium.

				Sample	
Time (hr.)	Flow rate (ml./hr.)	Volume (ml.)	Temperature (°C)	pH	Dry wt. organism (μg./ml.)
0	36	324	37·7	—	—
18	34·8	318	39·2	6·4	120
23	35·4	314	38·4	6·6	123
30	35·5	314	39·1	6·5	113
40·3	35·8	318	37·2	6·4	120
41·75	L-Arginine added to reservoir				
45·75	—	—	—	6·5	147
47·5	—	—	—	6·6	168
49·7	—	—	—	6·6	197
50·2	35·4	318	39·0	—	—
52·7	—	—	—	6·8	222
54·8	—	—	—	6·7	222
64·5	—	—	—	6·8	217
65	37·2	318	37·8	—	—
69·25	—	—	—	6·8	213
76·75	—	—	—	—	224
78·25	—	—	—	6·8	220
79·75	37·1	318	37·2	—	—

Table 2. *Growth of* Streptococcus faecalis *on* D-(^{14}C)-*glucose*

Organism grown in 150 ml. of partially defined medium containing 150 mg. D-(^{14}C)-glucose (Specific activity = 838 counts/min./mg. glucose). Incubation temperature = 37°. At the end of growth the dry wt. organism/ml. culture was measured turbidimetrically (Spekker Absorptiometer) and organisms harvested and prepared for radioactive assay as described in the text.

Dry wt. organism (mg.)	Glucose added (c.p.m.)	Radioactivity of organisms (c.p.m.)	Glucose incorporated (% of that added)
27·8	125,700	1,156	0·92

suspension was then put through the washing procedure and the radioactivity of the organisms measured; the activity was no greater than background. Table 2 shows the results of the experiment. The organisms produced weighed 27·8 mg. and the total radioactivity was 1156 counts/min., equivalent to 0·552 mg. glucose-carbon, or 0·92 % of the glucose added. If the carbon content of the cells is taken as 50 % of the dry wt. then the substrate supplied 4 % of the cell-carbon.

DISCUSSION

Table 3 lists the values of Y we have obtained for *Streptococcus faecalis, Saccharomyces cerevisiae, Pseudomonas lindneri* and *Propionibacterium pentosaceum.* Since the ATP yields of the catabolic reactions used by the first three of these organisms are known we also list in Table 3 the values for Y^{ATP} calculated from the various yield coefficients. The mean of the 8 values for Y^{ATP} is 10·5 (range = 8·3–12·6). Considering the fact that these organisms, *S. faecalis, S. cerevisiae* and *P. lindneri*, belong to quite different taxonomic groups, the agreement between the values is striking. We conclude from these results that, under the cultural conditions used, i.e. anaerobic growth on complete media with the energy source as the limiting factor, the yield of these three organism/mole of ATP is constant. From this it follows that the amount of biologically useful energy these organisms obtain per mole of energy source is more precisely expressed by the ATP yields of their respective catabolic reactions than by the standard free energy changes computed for these reactions. The operative phrase here is 'biologically useful energy' by which we mean that amount of energy made available by a catabolic process which the cell is able to use for the performance of the chemical, osmotic and mechanical work associated with growth. So far only three organisms have been studied and there is clearly a need for further investigations. The main requirement is for organisms with catabolic processes the ATP yields of which are known with certainty. That the ATP yield of a catabolic reaction rather than the free energy change is a more accurate index of the amount of biologically useful energy produced by that reaction is shown very clearly by the results with *S. cerevisiae* and *P. lindneri.* Both of these organisms convert glucose anaerobically to ethanol and CO_2 along with some minor end products. The yeast ferments glucose by the Embden–Meyerhof pathway with an ATP yield of two, whereas *P. lindneri* catabolizes glucose via 2-oxo-3-deoxy-6-phosphogluconate (the Entner–Doudoroff pathway) with an ATP

Table 3. *Growth yields of* Streptococcus faecalis, Saccharomyces cerevisiae, Pseudomonas lindneri *and* Propionibacterium pentosaceum

The values given are the means of all determinations shown in Figs. 1–4 and 6.

Organism	Medium	Substrate	ATP yield (mole ATP/ mole substrate)	$Y_{\text{substrate}}$ (g. dry wt. cells/mole substrate)	Y^{ATP} (g. dry wt. cells/mole ATP)
S. faecalis	Partially defined	Glucose	2·0	22	11
S. faecalis	Complex	Glucose	2·0	23	11·5
*S. faecalis**	Complex	Glucose	2·0	18·5	9·3
S. faecalis	Complex	Ribose	1·67	21	12·6
S. faecalis	Complex	Arginine	1·0	10	10
S. faecalis†	Partially defined	Arginine	1·0	10·5	10·5
S. cerevisiae	Partially defined	Glucose	2·0	21	10·5
P. lindneri	Complex	Glucose	1·0	8·3	8·3
P. pentosaceum	Complex	Glucose	—	37·5	—
P. pentosaceum	Complex	Glycerol	—	20	—
P. pentosaceum	Complex	DL-lactate	—	7·6	—

* = values taken from Sokatch & Gunsalus (1957).
† = results from continuous culture experiments.

yield of one. Since the overall reaction is the same in both cases the free-energy changes will also be identical, regardless of the fact that the mechanisms involved differ markedly from one another. From this it follows that if the ATP yield is taken as the index of the amount of biologically useful energy made available to the organism then the value of Y_{glucose} of *S. cerevisiae* will be twice that of *P. lindneri*; whereas, if the free energy change is the index, then Y_{glucose} should be the same for both organisms. Table 3 shows that the Y_{glucose} of *S. cerevisiae* is somewhat more than double that of *P. lindneri*.

Since the amount of ATP produced by a growing culture cannot be measured directly we have assumed in calculating the values for Y^{ATP} that all the substrate added to the medium was catabolized. In the case of *Streptococcus faecalis* direct measurement of the amount of glucose-carbon assimilated using (^{14}C)-glucose showed that only 0·92 % of that added to the medium was assimilated. This amount was equivalent to some 4 % of the cell carbon. The result was not surprising since the complex nutritional requirements of *S. faecalis* indicate a very low synthetic ability. It was also known from the work of Smith & Sherman (1942) that growing cultures of this organism convert 90–95 % of the glucose in the medium to lactic acid.

The nutritional requirements of the other organisms studied are simpler than those of *Streptococcus faecalis*, indicating that when they are grown upon their respective minimal media significant amounts of substrate are assimilated. When assimilation occurs, the amount of substrate catabolized cannot be taken as equal to the amount of substrate which disappears from the medium. However, these organisms were, for the purpose of our experiments, grown on complex media and we assume that, under these conditions, their synthetic powers were repressed and that they utilized the preformed amino acids and other essential cell constituents present in their respective media. The validity of this assumption has not been tested with these organisms, but it is known from work with other microbes, in particular with *Escherichia coli* that repressions of this sort occur: for a review of the available evidence see Magasanik, 1957; Roberts *et al.* 1955.

Table 3 shows that when *Streptococcus faecalis* was grown in a complex medium the value for Y_{glucose} was, within experimental error, the same as that obtained when this organism was grown in the partially defined medium. The value for Y_{arginine} obtained by growing *S. faecalis* in continuous culture on the partially defined medium supplemented with a small amount of glucose was 10·5, which agrees well with the value of 10 obtained by growing the organism in batch culture on a complex medium + arginine. The ratio $Y_{\text{glucose}}/Y_{\text{arginine}}$ calculated from our results = 2·19 and is approximately equal to the ratio of the ATP yields from glucose and arginine, respectively, viz. 2·0.

Fukui, Ôi, Ôbayashi & Kitahara (1957) found that growing cultures of *Streptococcus faecalis* fermented both D-ribose and L-arabinose to lactate; small amounts of formate but no acetate were also produced. Washed suspensions prepared from cultures grown on arabinose converted this pentose almost quantitatively to lactate. To account for these results they suggested that both ribose and arabinose are first converted to hexose by the pentose cycle enzyme system and the hexose so formed fermented via the Embden–Meyerhof pathway. Sokatch & Gunsalus (1957) came to a similar conclusion as a result of their study of the fermentation of gluconate by *S. faecalis*. The ATP yield when pentose is fermented by this mechanism is 1·67 mole ATP/mole pentose (Elsden & Peel, 1958). The value we obtained for Y_{ribose} was 21 which gives $Y^{\mathrm{ATP}} = 12{\cdot}6$, a value somewhat greater than those calculated from the results with arginine and glucose.

It will be seen from Table 3 that the value of Y_{glucose} for *Propionibacterium pentosaceum* is approximately twice that of *Streptococcus faecalis* and *Saccharomyces cerevisiae* and four times that of *Pseudomonas lindneri*. If our conclusion that the growth of an organism is a function of the ATP yield of its catabolic processes is correct then it follows that *P. pentosaceum* synthesizes twice as much ATP per mole of glucose as either *S. faecalis* or *S. cerevisiae*, that is to say, the ATP yield from the fermentation of glucose by *P. pentosaceum* is 4 mole/mole glucose. The origin of this extra ATP is a matter of some interest. The fermentation of glucose is approximately described by reaction:

$$1{\cdot}5 \text{ glucose} \rightarrow 2 \text{ propionate} + \text{acetate} + 1CO_2.$$

There is evidence which suggests that the Embden–Meyerhof mechanism plays an important part in the fermentation of glucose by *P. pentosaceum* (Wood, Stjernholm & Leaver, 1955) and that pyruvate is the precursor of the end-products propionate, acetate and CO_2. If this is the case it follows that the conversion of 1·5 mole glucose to three mole pyruvate will yield 3 mole ATP. But the value for Y_{glucose} requires the formation of 6 mole ATP from 1·5 mole glucose, of which only three have so far been accounted for. The formation of acetate from pyruvate should give 1 mole ATP/mole acetate and, if this is correct, we are forced to conclude that the remaining 2 mole ATP arise from the reactions concerned with the formation of propionate. In other words, the formation of 1 mole of propionate is associated with the formation of one mole of ATP. It will be possible to test this prediction once the individual reactions in the synthesis of propionate have been defined and the enzymes responsible isolated.

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Some Properties of a Gram-Negative Heterotrophic Marine Bacterium

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SUMMARY

Some properties of a marine bacterium are described. This organism belongs to a large, but ill-defined, group comprising Gram-negative rods with no apparent action on monosaccharides. It is a polar flagellate, has limited acid tolerance, consistent with its inability to produce acid from sugars and has no constitutive system for metabolizing glucose. A weak inducible system was available, however, which enabled adapted organisms to grow on glucose and use it as a respiratory substrate. The organism also lacks a constitutive citric acid cycle but ability to metabolize succinate was induced by growth on succinate or glucose. It cannot use inorganic nitrogen for growth. Of the compounds examined, amino acids were the most generally satisfactory sources of energy, carbon, and nitrogen. The organism accumulated a relatively large pool of intracellular amino acids which probably contributed largely to its high rate of endogenous respiration. The classification of the bacterium is discussed.

INTRODUCTION

A Gram-negative heterotrophic marine bacterium, recently isolated from eastern Australian waters, was selected for the study of some aspects of its biochemistry and physiology with particular reference to its macro-molecular components. To facilitate reference to the organism in the future and because its classification raises some problems, some general properties are described in the present paper. The organism belongs to a group of bacteria which in some environments is probably of considerable numerical importance. This group contains Gram-negative rods without apparent action on the commoner hexoses and pentoses. In this category are to be found bacteria which are non-motile, motile with peritrichous flagella, and motile with polar flagella. Descriptions of bacteria of the first two kinds are listed with the Achromobacteriaceae (*Bergey's Manual*, 1957), while descriptions of the polar flagellates are included in the Pseudomonadaceae and the Spirillaceae. The present organism is of this third kind, being motile by means of a single polar flagellum. The organism was chosen for a number of reasons; like many Gram-negative marine or halophilic bacteria it is very pleomorphic, it is readily susceptible to osmotic lysis (which offers a means of rupturing cells without the disadvantages of mechanical or sonic disintegration) and it is readily autolysed (suggesting the presence of enzyme(s) which might be useful in degrading the cell wall and associated structures). These properties suggested that the cell wall of the organism is weak and that its study might provide information of a kind less readily obtainable from other sources. These and other aspects of the organism will be discussed elsewhere.

The properties examined were chosen (i) to give enough information about the organism to enable it to be recognized again; (ii) to give information about the principal mechanisms by which the organism obtains, or may obtain, its nutrients and energy. Under (i) is included the qualitative chemical composition of the cell wall. While there are as yet insufficient data to say whether cell-wall composition of the Gram-negative bacteria has any taxonomic significance, as it has for some Gram-positive genera (Cummins & Harris, 1956, 1958), it is undoubtedly one of the more suitable properties of most bacteria. Under (ii) emphasis has been placed on energy-yielding mechanisms as there is good reason for regarding this kind of activity as one which provides one of the more rational biochemical bases for classifying organisms in a broad sense and for providing a background against which to consider their natural relationships to each other.

METHODS

Source and maintenance of the bacterium. The bacterium was isolated from a water sample taken about 5 miles off Port Hacking, N.S.W., Australia, from a depth of 50 m., about 10 m. above the sea-bed. It has been maintained with weekly transfers on sea water peptone agar (see below) at 20°. Attempts to freeze-dry cultures were unsuccessful, reconstitution of the dried material in a variety of solutions being accompanied by complete dissolution of the solid components of the cells.

Media. Media were prepared in sea water (collected from the sea or the Port Hacking estuary) or in an artificial sea water of the following composition (per l.): NaCl, 24 g.; $MgCl_2$ $6H_2O$, 11 g.; Na_2SO_4, 4 g.; KCl, 0·7 g.; $CaCl_2$, 5 ml. of a 10% (w/v) solution added with stirring; Na_2HPO_4, 15 ml. of 0·2 M solution added with stirring; $FeSO_4.7H_2O$, 0·04 mg. Buffered artificial sea water contained phosphate buffer as described with the results.

Sea-water peptone and sea-water peptone agar contained per l., 10 g. Difco peptone and 2 ml. 0·2 M-Na_2HPO_4; the media were adjusted to pH 7·2–7·4. Such media were sterilized by autoclaving at 10 lb./in.^{2} for 5–10 min. With Difco peptone, no precipitation occurred under these conditions. The compositions of various defined media, which were sterilized by filtration through Millipore membrane filters, are given with the appropriate results.

Unless designated 'stagnant', all cultures were grown with aeration. Cultures were grown at room temperature (20–25°) unless otherwise stated. The term 'lag' in reference to growing cultures means the number of days from inoculation to the appearance of turbidity.

Cell-wall preparation and analysis. Cell walls were prepared by mechanical breakage in the Mickle disintegrator or by osmotic lysis followed by differential centrifugation (Salton & Horne, 1951) and in some instances by tryptic digestion (Cummins & Harris, 1956). Amino acid and amino sugar composition of cell walls was established by reaction with ninhydrin on 2-dimensional paper chromatograms run in 80% (v/v) aqueous pyridine and butan-1-ol + acetic acid + water (6:1:2, v:v:v) following hydrolysis in 6 N-NCl for 16 hr. at 100°. Sugars were recognized as described with the appropriate results following hydrolysis in 2 N-HCl for 2 hr. at 100°. Polyols (see Armstrong *et al.* 1959) were detected on paper chromatograms with the periodate + Schiff's base spray reagent of Buchanan, Dekker & Long (1950) after hydrolysis of cell walls as described with the results. Amino sugars were estimated by the method of Rondle & Morgan (1955) following hydrolysis of the cell wall for 2 hr. in 2N-HCl at 100°.

Miscellaneous methods. Intracellular amino acids were estimated by the method of Cocking & Yemm (1954). Determination of the mass of organisms for reference purposes in the estimation of amino acid pools and calculation of respiratory quotients was complicated by the need to wash them in salt or sucrose solutions to prevent osmotic lysis and by the fact that a significant amount of autolysis always occurred in the centrifuge, even in the presence of various protecting solutions. Amino acid pools and respiratory quotients were therefore referred to the weight of material precipitated in the cold by 5 % (w/v) trichloroacetic acid (TCA) and washed once with water. The precipitate was dried *in vacuo* over H_2SO_4. Checks on a terrestrial species of *Pseudomonas* treated in this way gave a ratio of dry weight of whole organisms: dry weight of TCA precipitate of 1:1·02.

Determinations of O_2 uptake were made at 25° (unless indicated otherwise) by conventional Warburg manometric methods.

Succinate was determined in artificial sea water media as follows. Sulphate and phosphate were removed by addition of $BaCl_2$ and cations were removed on Amberlite IR-120(H). The solution was concentrated in a stream of hot air and finally evaporated to dryness under vacuum in the presence of NaOH. In this way most of the Cl^- was removed. The material was then chromatographed on a strip of Whatman 3 MM paper in butan-1-ol + acetic acid + water (6:1:2, v:v:v) in which the R_f of succinic acid is about 0·73 and well ahead of the other anions present. The succinic acid was located by side markers, the strip plus suitable blanks and standards was cut out and soaked in a convenient volume of water. After about 30 min. the filter paper suspension was titrated directly with 0·01N-NaOH. The recovery of succinic acid by this method was about 96 %.

Glucose was estimated in artificial sea water media by the method of Somogyi (1952) after deionization with Amberlite IR-120(H) and IR-4B(OH).

Flagella were stained by the method of Leifson (1951) for which purpose it was necessary to suspend the organisms in sea water or an equivalent to prevent lysis. Other techniques are described with the appropriate results.

RESULTS

Cellular morphology. The organism in the logarithmic growth phase in an adequate medium showed rods with round ends, occurring mainly singly and in pairs. Under these conditions cellular dimensions were mainly within the range 1 × 1·5–2μ with a minority of organisms as small as 1 × 1·2μ and as large as 1 × 3·5μ. Under other conditions of growth, e.g. in old cultures, a variety of shapes and sizes might be present. These variations included spherical forms of diameter from 1 to 5μ, filamentous organisms and a variety of irregular shapes, illustrating the pleomorphism frequently attributed to marine bacteria (ZoBell, 1946). Logarithmic-phase organisms were motile by means of a single polar flagellum and motility was frequently retained by the irregular forms. The organism is Gram-negative.

Colonial appearance. On sea water + peptone agar, colonies 1 to about 7 days old were circular, raised, slightly undulate, smooth and translucent. They might be off-white or yellow which in older cultures deepened to orange-red. On subculture, white and yellow colonies were maintained 'pure' for a time but eventually reverted to a mixture of both types. Conditions leading to the appearance of one kind or the other were not investigated. In addition to the yellow pigment, a diffusible brown to black pigment was formed in most media and under favourable conditions darkened the medium considerably. This pigment was shown to be melanin by incubating a suspension of the organisms with tyrosine, or better, by preparing the enzyme tyrosinase. This was done by extracting acetone- or freeze-dried organisms with water or phosphate buffer (M/15, pH 7). Partial purification was achieved by precipitating with saturated $(NH_4)_2SO_4$, dissolving the precipitate in water and adding acetone to a final concentration of 40 % (v/v). The acetone precipitate was discarded and the supernatant fluid which contained the enzyme was dialysed against running tap water. All these processes were carried out satisfactorily at 20°; frozen solutions of the enzyme were stable indefinitely. The activity of the enzyme was conveniently assayed by following the development of the pink intermediate substance (absorption maximum 475 mμ) formed by the action of the enzyme on tyrosine. The activity measured in this way was greatest near pH 5·5 but subsequent polymerization to melanin was faster at more alkaline pH values. As with tyrosinases from other sources, the enzyme required Cu^{++}. Preparations from organisms grown in the presence of tyrosine or phenylalanine were 20–40 times more active than preparations from organisms grown without either of these amino acids.

Cell-wall composition. The amino acid composition of the cell wall is typical of Gram-negative bacteria, comprising, α,ϵ-diaminopimelic acid (DAP) and most of the amino acids normally found in protein hydrolysates. Amino sugars found were hexosamine and trace amounts of muramic acid (barely detectable with ninhydrin on paper chromatograms of hydrolysates of 2 mg. cell wall). Glucose was identified by reaction with aniline phthalate following paper chromatography in the butanol + acetic acid solvent, butan-1-ol + pyridine + water (6:4:3, v:v:v) and ammoniacal water-saturated phenol. The presence of a heptose was established by the sulphuric acid-cysteine reaction of Dische (1953). According to the criteria of Davies (1957) this is thought to be either D-glycero-L-galacto- or D-glycero-D-galactoheptose. Spots corresponding to glycerol and some unidentified polyols were detected on paper chromatograms of acid hydrolysates (2N-HCl at 100° for 2 and 22 hr. and 0·5N-H_2SO_4 at 100° for 20 hr.) of cell walls run in propan-1-ol + ammonia (15N) + water (6:3:1, v:v:v) and the butanol + acetic acid solvent system. Cell walls from organisms which had been grown on peptone contained 1·4–1·6 % amino sugar (as glucosamine) but this value could be altered by varying the nutrient composition of the growth medium as will be described elsewhere.

Miscellaneous biochemical properties. Antibiotic sensitivity (after Shewan, Hodgkiss & Liston, 1954) was examined by using 'Sentest' tablets (Evans Medical Supplies Ltd.). The organism was resistant to penicillin (2·5 international units) but sensitive to streptomycin (80 μg.) and chloramphenicol (100 μg.). Nitrates were reduced to nitrites. Gelatin was liquefied. Catalase was not formed. Acid was not produced from glucose. Ammonia was liberated during growth on peptone.

Miscellaneous biophysical properties. The organism grew over the approximate temperature range 10–40°. In an artificial sea water medium containing (g./l.) DL-alanine, 2·0; glycine, 3·0; DL-lysine.HCl, 0·4; DL-methionine, 0·4; L-leucine, 0·4;

L-arginine.HCl, 0·4 the growth rate was maximal at 35° (measured at 5° intervals), with a mean generation time of 1·85 hr. Appreciably higher growth rates occurred in media containing peptone. At about 40° (40·4°) in this defined medium, the organism grew arithmetically instead of exponentially with the formation of filaments and variety of grotesque cell shapes. At temperatures below about 10° growth ceased, the organisms lysed and, when tyrosine was present, additional amounts of melanin were formed on lysis.

The ability of the organism to grow in artificial sea water + peptone adjusted at intervals of 0·5 pH units over the range pH 6·0–9·0 was examined. Growth was greatly inhibited at pH 6·5, completely suppressed at pH 6, but occurred normally at pH 7 and above. In a series of transfers through progressive dilutions of artificial sea water containing 1 % (w/v) peptone, the organism, shortly after isolation, grew on the equivalent of 36 % (v/v) artificial sea water but not at 24 % or lower; after about 1 year in culture it grew on 10 % (v/v) artificial sea water, but not at 5 % or lower. It also grew readily in double strength artificial sea water containing 1 % peptone. More concentrated media were not investigated.

Table 1. *Ability of the marine bacterium to use selected amino acids* (1·0 %, *w/v*) *and amino acid mixtures* (*total concentration* 1·0 %, *w/v*) *for growth in stagnant culture in artificial sea water*

No growth	Poor growth	Average growth	Good growth
α,ϵ-Diaminopimelic acid	Glycine	DL-Alanine	L-Glutamate
L-Tryptophan	DL-Phenylalanine	L-Serine	Alanine + arginine
	L-Tyrosine	L-Arginine	Serine + arginine
		L-Aspartate	
		Alanine + serine	

Nutrition. The nutritional requirements of the organism are simple but restricted. In general, amino acids were found to be adequate sources of carbon and nitrogen. Table 1 shows its ability to use selected amino acids and amino acid mixtures for growth. Growth on tyrosine or phenylalanine was slow and total crops were low but the organisms were of normal appearance. Growth on glycine did not always occur; total crops were very low (in stagnant cultures maximal optical density at 700 mμ of *c.* 0·04), many of the organisms were grossly distorted and about 2 days after the appearance of turbidity such cultures underwent extensive lysis, no visible turbidity remaining.

Serine produced more rapid growth than alanine but a similar total crop. Aspartate and glutamate (singly) gave variable results; sometimes no growth occurred at all. The lag in stagnant cultures (inoculum, stationary phase sea-water peptone culture, 1 loop/10 ml.) was about 4 days with glutamate, compared with about 1 day for the amino acids in column 3 of Table 1.

Preliminary observations showed that no growth occurred in the presence of NH_4^+ plus any of the following compounds: galactose, glucose, fructose, rhamnose, ribose, acetate, citrate, lactate, pyruvate or succinate. Selected carbon sources were then tested for their ability to stimulate growth in the presence of low concentrations of arginine (0·025 %, w/v, of monohydrochloride). Under these conditions citrate, fructose and glycerol had no effect; glucose, galactose and lactate hastened the appearance of turbidity and caused slight increases (*c.* 20 % in stagnant cultures) in total crop, while succinate markedly hastened the appearance of turbidity and increased the total crop. Chemical determinations showed disappearance of succinate (see below), failed to show any disappearance of glucose (see below); they were not made for lactate and galactose. An additional effect of glucose was apparent in older stagnant cultures. The total maximal crop on arginine + glucose was only about 20 % more than on arginine alone but whereas the arginine control cultures subsequently underwent extensive lysis, the arginine + glucose cultures did not. The conditions were such that this was unlikely to be an osmotic 'stabilizing' effect.

With succinate or lactate as carbon source hydroxylamine, like ammonia, did not provide nitrogen for growth, but urea was readily used in conjunction with succinate after a lag of about 3 days (inoculum, 1 loop of stationary phase peptone culture/10 ml.).

The ability of the organism to use glucose was confirmed as follows. Buffered artificial sea water (0·025M-phosphate buffer, pH 7·2) containing urea (0·25 %, w/v) and glucose (0·5 %, w/v) was inoculated with 2 loops/10 ml. of stationary phase cultures grown in the urea + succinate or arginine + glucose media. The organisms grown on arginine + glucose grew on urea + glucose after a lag of 1 day (in stagnant culture). Organisms grown on urea + succinate grew on urea + glucose after a lag of 10 days. In neither case was measurable acid produced in this medium. The organism did not grow on urea alone.

Respiratory substrates

Unless indicated otherwise, organisms were grown for manometric observations in 1·5 l. sea water + peptone for 24 hr. at 30°. They were washed twice and resuspended in artificial sea water + 0·05M-phosphate buffer (pH 7). Such organisms had a marked pink (cytochrome) colour and a high but somewhat variable rate of endogenous respiration which, after vigorous aeration for 4 hr. in buffered artificial sea water at 20–25°, could be decreased to about 60 % of its former value. Osmotic lysis of freshly harvested organisms decreased the endogenous respiration rates by about 15 % and the respiration rate of the lysed organisms was not appreciably different whether they were suspended in 0·05M-phosphate buffer or in buffered artificial sea water.

On the other hand, respiration rates in the presence of oxidizable substrates were not high and the degree to which respiration was increased was markedly affected by the conditions under which the organism had been grown. Figure 1 shows respiration rates of the organism compared with those of a species of *Pseudomonas* (Brown, 1957) grown under approximately similar conditions, namely, 24 hr. at 30° in 1 % (v/v) sea water containing 1 % (w/v) peptone.

Table 2 gives a list of substrates and the degree to which they stimulated respiration when used under the conditions of Fig. 1.

Amino acids. Alanine, which was one of the better substrates, was more obviously effective in organisms whose respiration had been decreased either by aeration in buffered artificial sea water or by growth in 50 % (v/v) sea water + peptone, which decreased endogenous respiration by about 45 %. Under these conditions increases in the absolute rates of oxygen uptake with alanine were also noted; the overall effect of the substrate was to stimulate respiration rate by about 200 %.

It was thought that the relatively high endogenous respiration might be reflected in the intracellular amino acid pools; these were determined, therefore, on the marine organism and for comparison on the pseudomonad. For this purpose the bacteria were grown under the conditions of Fig. 1. The marine organism was washed twice in 0·5M-sucrose containing 0·025M-phosphate buffer (pH 7) freeze-dried and extracted with suitable volumes of cold 5 % (w/v) TCA. The TCA was removed with

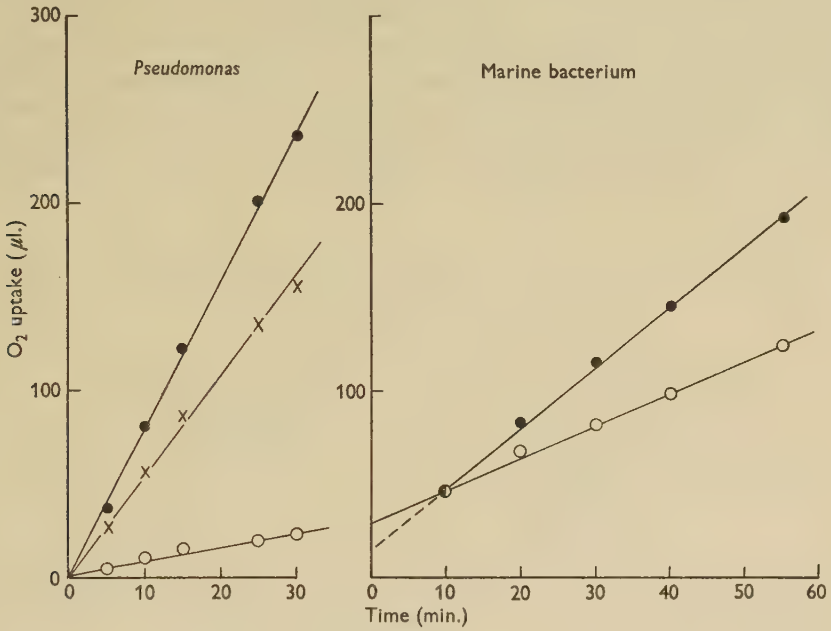

Fig. 1. Rates of oxygen uptake at 25° by the terrestrial psychrophilic *Pseudomonas* sp. in the presence of DL-alanine or glucose and by the marine bacterium in the presence of DL-alanine. Flasks contained cells (equivalent to 2·3 mg TCA precipitate/ml. buffered artificial sea water), 1·00 ml.; 0·5M-phosphate buffer, pH 7, 0·25 ml.; substrate (0·1M, or 0·05M-NaCl for endogenous), 0·25 ml.; KOH in centre well. Absolute values of oxygen uptake are given, no correction being made for endogenous respiration. (Alanine, ●; glucose, ×; endogenous, ○.)

Table 2. *Ability of the marine bacterium, after growth on peptone, to use selected compounds* (0·017M) *as respiratory substrates*

The figures represent the percentage increase of respiration above the endogenous rate at 25°

DL-Alanine	44–80	Pyruvate	120	Glucose	0
L-Aspartate	36–53	Lactate	53	Galactose	0
L-Glutamate	53	Succinate	0*	Glucosamine	0
Glycine	25 (at 29°)	Fumarate	0*	NH$_4^+$	−84 (inhibition)
L-Serine	180	Malate	0		
		α-Ketoglutarate	0		
		Acetate	17		
		Glyoxylate	0		
		Glycollate	34		

* On one occasion succinate caused an increase of 47 % after a lag of 190 min. On another occasion fumarate caused an increase of 180 % after a lag of 120 min.

ether from the extract before amino acid analysis. (During harvesting and washing some of the organisms autolysed and the 'curd' of cell debris and viscous material (deoxyribonucleic acid) which collected above the pellet in the centrifuge tube was always discarded.) The pseudomonad was washed twice in 0·05 M-phosphate buffer (pH 7), freeze-dried and treated as above.

The marine bacterium contained 350 μmole glutamic acid equivalents/g. TCA precipitate compared with 100 μmole/g. for the pseudomonad. The identity of the amino acids in the extracts of both organisms is given in Table 3.

Citric acid cycle intermediates. The failure of citric acid cycle intermediates to stimulate respiration (except in the two cases of inducible utilization; Table 2) might imply the absence of this pathway from the marine organism. To establish whether or not such negative findings were caused by permeability effects, measurements of oxygen uptake rates in the presence of succinate were made on osmotically-lysed organisms. The organism was grown in sea water + peptone, harvested and washed as described above, lysed by suspending in cold 0·25 M-phosphate buffer (pH 7) mixed with an equal volume of double strength artificial sea water containing 0·025 M-phosphate buffer and diluted to the required concentration with buffered artificial sea water. The preparation of lysed organisms had no action on succinate. It seems improbable that excessive disorganization of electron transport systems occurred under these conditions, since the endogenous respiration remained at 85 % of the rate for whole organisms (under Anoptral phase contrast all organisms were judged to have lysed) and respiration rates in the presence of aspartate were decreased only slightly by lysis.

Table 3. *Free amino acids in TCA extracts of the marine bacterium and the pseudomonad*

Marine bacterium	Pseudomonad
Alanine*	Alanine†
Aspartic acid	Aspartic acid
Glutamic acid	Glutamic acid
Leucine/*iso*leucine	Lysine
Lysine	
Phenylalanine	
Proline	
Valine/methionine	

* These amino acids accounted for nearly all the absorption at 570 mμ, compounds other than free amino acids contributing only to a minor extent. Aspartic acid, glutamic acid and alanine were the major components, while lysine was present in trace amounts.

† Glutamic acid was the predominant amino acid and was responsible for about 36 % of the absorption at 570 mμ (determined by eluting ninhydrin spots from the paper chromatogram with 75 % (v/v) aqueous acetone). Aspartic acid and alanine contributed about 5 % and 3 % respectively. The major component was not a free amino acid, or at least not a known amino acid. It was not identified and represented about 52 % of the total absorption. The remaining 4 % of the total absorption was due to traces of unidentified compounds.

Measurements of respiration rates in the presence of succinate and other substrates were made after growth of the organism under the following conditions: (1) 24 hr. at 30° in sea water + peptone containing 0·5 % (w/v) succinic acid (disodium salt); (2) 45 hr. in phosphate-buffered (0·025 M, pH 7) (v/v) artificial sea water containing arginine (0·05 %, w/v; monohydrochloride) and succinate (0·1 %, w/v, succinic acid); (3) 7 hr. at 30° from a heavy inoculum in 500 ml. 50 % (v/v) buffered artificial sea water containing arginine (0·025 %, w/v; monohydrochloride) and 0·004M-succinate (0·047 %, w/v, succinic acid). The inoculum was the centrifuged washed crop from 1·5 l. sea water + peptone medium grown overnight at 30°.

Organisms grown in the peptone medium showed no differences attributable to succinate. Succinate, fumarate, and malate each failed to stimulate respiration. After growth for 45 hr. in the arginine + succinate medium, 30 % of the succinate was used without concomitant formation of detectable quantities of other carboxylic acids, the organisms were white (no cytochrome colour), the endogenous respiration rate was about 38 % of that of organisms grown for 26 hr. on peptone, and neither succinate nor alanine had any effect on respiration.

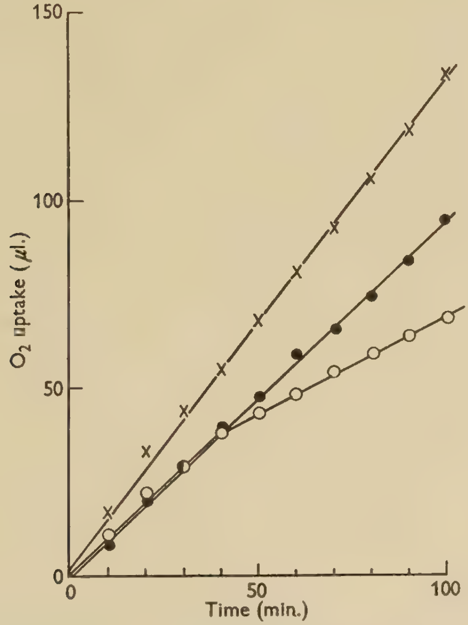

Fig. 2. Rates of oxygen uptake at 29° of the marine bacterium in the presence of DL-alanine or succinate. The organism was grown for 7 hr. in the arginine + succinate medium and conditions, other than temperature, were as described for Fig. 1. Aspartate and malate (not shown) had no effect on respiration and glutamate stimulated respiration to the same extent as alanine. (Alanine, ●; succinate, ×; endogenous, ○.)

When the heavy inoculum was incubated in the arginine + succinate medium, all the succinate was used within 7 hr. and no other carboxylic acids detectable on paper chromatograms accumulated. The respiration of such organisms was stimulated by succinate as shown in Fig. 2.

Glucose. The ability of glucose to stimulate the respiration of organisms grown in other than peptone medium was tested as follows.

(1) A heavy inoculum such as described above was incubated at 30° in buffered 50 % (v/v) artificial sea water containing arginine (0·025, w/v, monohydrochloride) and glucose (0·05, w/v). Glucose determinations were made on the supernatant

fluid sampled 3·5 and 7·5 hr. after inoculation. Measurements of respiration rates were made on organisms harvested at 7·5 hr. The following results and their standard deviations were obtained for the glucose determinations: initial sample, 0·468 ± 0 mg./ml.; 3·5 hr. sample, 0·436 ± 0·010 mg./ml.; 7·5 hr. sample, 0·444 ± 0·023 mg./ml. Glucose and succinate had no effect on the respiration of the organisms harvested after 7·5 hr., while alanine and acetate caused increases of similar magnitude to those listed in Table 2.

(2) The centrifuged crop from an overnight 30° culture (1 l.) of the organism in sea water + peptone medium was suspended in *c.* 200 ml. of buffered artificial sea water containing arginine (*c.* 0·05 %, w/v; monohydrochloride) and glucose (*c.* 0·05 %, w/v). After 24 hr. at 30° determinations were made of glucose concentration in the culture fluid and of respiration rates in the presence of selected substrates. The following concentrations of glucose were found: initial sample, 0·612 ± 0·055 mg./ml.; 24 hr. sample, 0·606 ± 0·024 mg./ml.

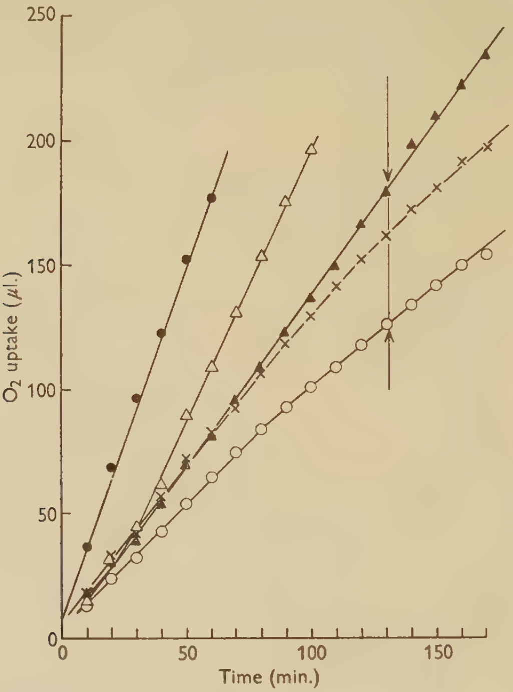

Fig. 3. Respiration rates at 26·5° of the marine bacterium after incubation for 24 hr. in a growth medium containing arginine and glucose. Values of oxygen uptake obtained in the presence of the added substrates, acetate, DL-alanine, glucose and succinate have not been corrected for the endogenous values. The arrow marks addition of iodoacetate to a final concentration of 0·001 M. Other conditions were described for Fig. 1. (Acetate, △; alanine, ●; glucose, ×; succinate, ▲; endogenous, ○.)

The manometric results are shown in Fig. 3, from which it will be seen that both glucose and succinate caused a slight stimulation of respiration and acetate caused an increase of 113 % over the endogenous rate (cf. Table 2). The slight change in slope of the glucose curve following addition of iodoacetate represents an inhibition of 40 % of the rate of oxygen uptake after subtraction of endogenous values.

DISCUSSION

It is clear from the results that the marine bacterium lacks a constitutive means of metabolizing glucose and that its inducible mechanism is not sufficiently vigorous to cause the disappearance of glucose in quantities large enough to be detected under the experimental conditions used. The ability of glucose-trained organisms to grow on glucose + urea, however, leaves little doubt about an actual consumption of glucose as distinct from a catalytic or physico-chemical stimulus which might otherwise have been held to account for the effects observed with this sugar. Acid production from carbohydrates other than glucose was not investigated as it was considered that the very limited acid tolerance of the organism would lead to a negative result in all such tests.

It is also apparent that the organism has no constitutive citric acid cycle but does have an inducible system for metabolizing succinate. Whether or not this system involves an inducible citric acid cycle has not been investigated but the evidence of Fig. 3 suggests that it might. The enhancement of acetate metabolism by growth on glucose and the induction by glucose of succinate metabolism are, at least, consistent with such a hypothesis. It should be noted, on the other hand, that growth on succinate did not lead to simultaneous induction of a system for malate respiration (Fig. 2), but in this case no account was taken of permeability.

The effect of ionic conditions on these two inducible systems (glucose and succinate) was not investigated (cf. Payne, 1958) but the ionic composition of growth, washing and suspending media was maintained at a level found adequate for good growth and hence, presumably, for the synthesis of inducible enzymes. Similarly, beyond comparing endogenous respiration of lysed organisms in dilute phosphate buffer (containing Na^+ and K^+) and in artificial sea water, the effects of ionic conditions on respiration were not investigated (cf. Tomlinson & McLeod, 1957); the assumption was made that ionic conditions suitable for good growth would be suitable for a survey of respiratory substrates.

Of the compounds tested, amino acids were the most satisfactory sources of carbon, nitrogen and energy. Utilization of at least some amino acids (e.g. alanine) evidently involved constitutive enzymes, notwithstanding the 2 days old organisms which were unable to metabolize either alanine or succinate. Other 3-C amino and carboxylic acids, namely serine, pyruvic and lactic acids were also good respiratory substrates, while the 2-C amino acid, glycine was poor. Whole peptone-grown organisms cannot oxidize added glyoxylate (Table 2). If glycine is oxidized to glyoxylate as is likely (Kornberg & Sadler, 1960), a block at glyoxylate could explain the inadequacy of glycine as a single nutrient. The fact that some growth did occur on glycine is probably explained by the 'catalytic' activity of amino acids from the intracellular pool, and these amino acids also undoubtedly contributed to the high rate of endogenous respiration. It is of interest that growth in a medium of lower salt concentration decreased the endogenous respiration rate but not the total amount of pool amino acids extracted from a unit mass of TCA-precipitable material.

In attempting to classify the present organism the standard of reference and nomenclature used was *Bergey's Manual* (1957). As the classification of Gram-negative bacteria generally, and, in particular, genera of the Pseudomonadaceae,

Table 4. *A comparison of selected properties of the marine organism and other bacteria*

Unless indicated otherwise the properties of the named species are as listed in *Bergey's Manual.*

	Marine organism	*Vibrio hyphalus*	*Pseudomonas nigrifaciens*	*Halobacterium salinarium*	*P. aeruginosa*	*P. fluoroescens*
Salt requirements (growth)	(Sea water) > 0·025 M	(Sea water) *c.* 0·5 M	Opt. 0·26–0·43 M	> 2·7 M (opt. 4·8–5·5 M)	0–0·5 M*	0–0·5 M*
Use of inorganic N for growth	–	(Not stated)	(Not stated)	–	+*	+*
Acid from glucose	–	–	–	–	+ (Oxidative)	+ (Oxidative)
Liberation of NH_3 from peptone	+	+	+	(Not stated)	+*	+*
Reduction of nitrate	+	+	–	–	+	(Variable)†
Catalase	–	(Not stated)	(Not stated)	(Not stated)	+*	+*
Acid tolerance (growth)	> pH 6·5	(Not stated)	Opt. pH 6·8–8·4	(Not stated)	> pH 4·5*	> 4·5*
Temperature relations (growth)	*c.* 10°–40° opt. *c.* 35°	Opt. 20°–25°	4°–35°	Opt. 37°	10°–> 40°‡ (12°–30°)*	0°–30° (12°–30°)*

* Listed by Rhodes (1959) as part of a proposed definition of the genus *Pseudomonas.*

† Data of Rhodes (1959).

‡ Data of Brown (1957).

Spirillaceae, and Achromobacteriaceae is confused, however, this standard has been used with some reservations. Table 4 lists some properties of the present organism and similar organisms of three other genera from *Bergey's Manual*. Corresponding properties of *Pseudomonas aeruginosa* (the type species of the genus *Pseudomonas*) and *P. fluorescens* are included for comparison. The present organism is sufficiently similar in these and other properties to *Vibrio hyphalus* and *P. nigrifaciens* for it to be placed in either genus without causing any new anomalies. It is instructive to note that a selection of one of these genera in preference to the other involves a decision at the level of family and that, on the criteria of *Bergey's Manual* the decision must be made on whether or not, in the observer's opinion, the cells of the organism are curved. Gram-negative marine and halophilic bacteria are notoriously pleomorphic and it is doubtful whether the variations in size and shape which occur should be given any generic significance. Sea water is an effective stabilizing medium and it is reasonable to expect many Gram-negative bacteria which have evolved in marine or similar environments to have weak cell walls. This is evidently true of the present organism and the mechanical weakness of its walls is possibly reflected in their relatively low content of amino sugar (see Table 5). In this respect it is reminiscent of the spheroplasts to other Gram-negative bacteria.

Table 5. *Amino sugar content of the cell walls of the marine organism and other Gram-negative bacteria*

(Data for the other bacteria from Salton, 1958)

Organism	% amino sugar (as glucosamine) (2 N-HCl hydrolysates)
Marine organism	1·4–1·6*
Escherichia coli	2·4–4·0
Spirillum serpens	6·8
Chlorobium thiosulphatophilum	4·2
Rhodospirillum rubrum	2
Vibrio metchnikovii	1·9
Salmonella pullorum	4·8
Salmonella gallinarum	3·9
Gram-negative coccus LC1	13

* The values quoted here are those obtained from organisms grown on peptone and are more comparable with Salton's figures than values for cell walls of organisms grown on various defined media. Amino sugar contents of cell walls of organisms grown on defined media, which will be described elsewhere, fell within the range 0·97–2·5 %

If the salt requirements of the genus *Halobacterium* were less rigidly defined, this genus could provide a convenient name for the marine bacterium since, in respect of its production of orange (presumably carotenoid) pigment, inability to produce acid from carbohydrates or use inorganic nitrogen for growth, its oxidation of various amino acids, and, of course, its pleomorphism the marine organism resembles *H. salinarium*. The exclusion of the present organism from the halobacteria exemplifies a difficulty caused by delineating a genus by quantitative differences in a single physico-chemical property.

A classification of the organism must place it in the family Pseudomonadaceae. The selection of a genus is more difficult and in the writer's opinion is limited to the alternatives of (i) allocating the organism to the genus *Pseudomonas*, or (ii) creating a new genus. *Bergey's Manual* (1957) lists the genus *Pseudomonas* under section I of the family Pseudomonadaceae together with bacteria which 'attack glucose and other sugars either oxidatively or fermentatively'. The type species of the genus, *P. aeruginosa*, oxidizes glucose vigorously and will grow on inorganic nitrogen (plus a suitable source of carbon) as will *P. fluorescens*, a representative of a second important type of pseudomonad. While variations in any 'single' property must be expected within any defined group of organisms, a vigorous constitutive oxidative carbohydrate metabolism and an ability to grow on inorganic nitrogen carry such far-reaching implications about the biochemistry and physiology of the organisms, that when they are correlated with the other features of pseudomonads, namely Gram-negative staining, polar flagellation and an obligately aerobic physiology, they provide the basis of a precise and comprehensive definition which should not be lightly ignored. Hugh & Leifson (1953) have already discussed the taxonomic importance of oxidative as distinct from fermentative carbohydrate metabolism; Brown & Weidemann (1958) found that the use of this criterion, together with that of resistance to penicillin, provided the only effective means of classifying a number of non-motile species of *Pseudomonas*.

There is, of course, precedent for ignoring these properties. Having described the genus as quoted above, *Bergey's Manual* goes on to include in the genus species (e.g. *Pseudomonas nigrifaciens*, *P. gelatica* and *P. halestorga*) which are without apparent action on sugars (use of inorganic nitrogen is not recorded in these cases). The precedent is one which the present writer prefers not to follow, however, as it is the support of this practice which has produced the situation where the genus *Pseudomonas* may be logically distinguished from *Vibrio* only by a minor difference in the shape of the cell. The writer feels inclined therefore to advocate either the removal of all barriers so that all Gram-negative polar flagellates are called by the same generic name, or perhaps more reasonably, the creation of a new genus which would include the present organism and others e.g. *Vibrio hyphalus*, *P. nigrifaciens* and *Halobacterium salinarium*. It is, however, premature to formulate a definite proposal to this effect without an examination in the terms of this paper of a number of other similar organisms from various sources. It is hoped that further work will show whether such an opinion is justified. In the meantime the organism is left unnamed and will be referred to elsewhere by its number in the culture collection of these laboratories, marine pseudomonad no. 11.

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The Synthesis of Enzymes Concerned in Bacteriochlorophyll Formation in Growing Cultures of *Rhodopseudomonas spheroides*

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SUMMARY

The enzymes δ-aminolaevulic acid synthetase and δ-aminolaevulic acid dehydrase are concerned in the early stages of tetrapyrrole formation; factors controlling their synthesis have been studied in cultures of *Rhodopseudomonas spheroides* growing exponentially. In organisms growing in the dark under high degrees of aeration the differential rate of synthesis (increase in enzyme/increase in culture density) of both enzymes is about one-third of that in cultures growing anaerobically in the light. Organisms growing in the dark under low degrees of aeration form the enzymes at rates comparable to those in photosynthetic cultures. Under anaerobic conditions the differential rate of synthesis of both enzymes is decreased by increasing the light intensity. Enzyme synthesis in the light is repressed by oxygen, the effect being overcome on restoration of anaerobic conditions. Formation of bacteriochlorophyll under these various conditions is affected in the same way as enzyme synthesis. Addition of δ-aminolaevulic acid or haemin to growing cultures stopped the synthesis of the synthetase and the dehydrase; magnesium protoporphyrin had no such effect. The activity of the synthetase, but not of the dehydrase, was decreased in organisms growing with suboptimal concentrations of biotin. A component of the electron transport chain as well as the intracellular concentration of biosynthetic intermediates may contribute, by mechanisms as yet unknown, to the regulation of synthesis of enzymes involved in bacteriochlorophyll formation.

INTRODUCTION

Rhodopseudomonas spheroides and other members of the Athiorhodaceae are able to grow in the light or in the dark and there is a close connexion between the growth conditions and the formation of the photosynthetic pigments, bacteriochlorophyll and carotenoids. These are synthesized by organisms growing anaerobically in the light but only traces are found in cultures grown in the dark under high aeration (van Niel, 1944; Cohen-Bazire, Sistrom & Stanier, 1957). *R. spheroides* can form pigments in the dark but only when the oxygen tension is decreased (Cohen-Bazire *et al.* 1957; Lascelles, 1959). The mechanism of adjustment of the pigment content to suit the environment is undoubtedly complex but the degrees of activity of enzymes concerned in pigment synthesis may contribute. This possibility has been examined in *R. spheroides* in the case of bacteriochlorophyll which is probably formed by a pathway which includes the intermediates shown in Fig. 1 (Lascelles, 1956*a*; Cooper, 1956; Kikuchi, Kumar, Talmage & Shemin, 1958; Gibson, 1958; Heath & Hoare, 1959; Hoare & Heath, 1959). It was found previously that the activities of δ-aminolaevulic acid (ALA) synthetase and ALA dehydrase were correlated with the ability to form bacteriochlorophyll, both enzymes being about five times more active in organisms grown photosynthetically than in those grown in the dark under high aeration (Lascelles, 1959). In the present work a study has been made of the influence of the environment, including the gas phase and constituents of the medium, on the rates of synthesis of these enzymes in exponentially growing cultures.

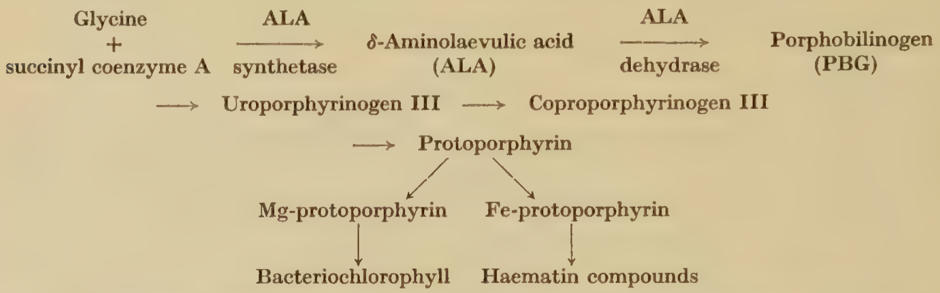

Fig. 1. Pathway of synthesis of tetrapyrrole derivatives in *Rhodopseudomonas spheroides*.

METHODS

Organism. The strain of *Rhodopseudomonas spheroides* (NCIB no. 8253) and the maintenance of stock cultures were described previously (Lascelles, 1956*a*).

Medium. All experiments were made with cultures growing in the chemically defined medium containing malate and glutamate and supplemented with iron citrate (0·01 mM) which has been described before (Lascelles, 1959).

Inoculation of cultures. To obtain exponentially growing cultures organisms were first grown in the above medium either anaerobically in the light or aerobically in the dark as described by Lascelles (1959). They were harvested by centrifugation at the end of the exponential phase of growth (about 16 hr.), when the density had reached 0·9–1·0 mg. dry wt. organism/ml. The centrifuged organisms were resuspended in fresh medium to a density equivalent to *c.* 0·2 mg. dry wt./ml. and then incubated under the appropriate conditions.

Conditions of incubation. Most experiments were made with cultures growing anaerobically in the light in Drechsel bottles (capacity 275 ml.); anaerobiosis, as well as mixing of the cultures, was maintained by continuously passing a gentle stream of 5 % (v/v) CO_2 in N_2. The culture bottles were incubated in a glass-sided water bath at 34°; each vessel was illuminated by a 100 W. tungsten lamp mounted in a reflector clamped outside the bath and 10 cm. from the centre of the culture. These conditions are referred to as 'standard'. In some experiments a high light intensity was used, Philips 'Photolita' no. 1 (275 W.) photoflood lamps being substituted for the 100 W. lamps. Other deviations from the standard conditions are noted in the text.

For aerobic incubation in the dark, cultures were grown in ⊥-shaped tubes similar to those described by Monod, Cohen-Bazire & Cohn (1951); the horizontal limb containing the culture was of 3·3 cm. internal diameter and 19 cm. long. These were clamped to a rocking device (see van Heyningen & Gladstone, 1953) and shaken at 36 oscillations/min. in a water bath at 34°. For growth under a high degree of aeration each ⊥-tube contained 50 ml. culture; conditions of low aeration were achieved by using 100 ml. culture/vessel, which almost filled the horizontal limb.

Estimation of growth, bacteriochlorophyll and protein. Growth was determined by measuring the extinction of cultures at 680 mμ with a Unicam spectrophotometer (SP-600); the dry wt. organism/ml. was calculated from calibration curves obtained with organisms grown either anaerobically in the light or aerobically in the dark. The protein content of the organisms was 50 % of the dry wt. by the method of Stickland (1951).

Bacteriochlorophyll in the organisms and protein in the cell-free extracts were estimated as described previously (Lascelles, 1959).

Preparation of culture samples for assay. At intervals samples were removed from the growing cultures for estimation of dry wt. of organisms, bacteriochlorophyll and enzymic activity. The volume removed depended on the culture density, varying from 25 to 100 ml., of which 6 ml. were used for determination of the dry wt. and bacteriochlorophyll. The rest of the sample, containing 15–20 mg. dry wt. organism, was centrifuged, the organisms washed in 0·02 M-2-amino-2-hydroxymethyl-propane-1:3-diol (tris) buffer (pH 7·4) and finally suspended in 4–5 ml. of 0·04 M tris buffer (pH 7·4). When necessary these suspensions were stored at −20° before preparation of the cell-free extracts for estimation of ezyme activity.

Preparation of cell-free extracts. The suspensions were disrupted by ultrasonic vibration for 2 min. at 600 W. and 25 kc./sec. with a Mullard ultrasonic generator type E7590B (Mullard Ltd., London, W.C. 1); the transducer assembly was cooled by immersion in a rapidly flowing stream of tap water. Whole organisms and debris were removed by centrifugation at 25,000 *g* for 10 min. at 0°. The supernatant fluid containing between 1 and 3 mg. protein/ml. was used in the assays for enzymic activity without further treatment.

Assays of enzymic activity. The enzyme system responsible for the condensation of glycine and succinyl coenzyme A to give ALA (referred to as ALA synthetase) was studied in partially purified preparations from *Rhodopseudomonas spheroides* by Kikuchi *et al.* (1958), Kikuchi, Kumar & Shemin (1959) and by Gibson (1958). It was assayed by a method modified slightly from that used previously (Lascelles, 1959). The reaction mixture contained: extract of organisms (0·6–1·5 mg. protein); tris buffer (pH 7·4), 50 μmole; $MgCl_2$, 12·5 μmole; glutathione, 1 μmole; coenzyme A (CoA), 0·05 μmole; pyridoxal phosphate, 0·1 μmole; adenosine triphosphate (ATP) 8 μmole; glycine and sodium succinate, each 100 μmole; water to 1·0 ml. The mixtures were incubated in 1·5 cm. diameter tubes for 2 hr. at 30° before stopping the reaction by adding 1 ml. of 10 % (w/v) trichloroacetic acid. The amount of ALA formed was estimated colorimetrically after condensation with aminoacetone by the method of Mauzerall & Granick (1956).

δ-Aminolaevulic acid dehydrase activity was estimated as previously described (Lascelles, 1959).

One unit of ALA synthetase or ALA dehydrase is defined as that amount which catalyses the formation of 1 μm-mole of ALA or porphobilinogen (PBG) respectively under the standard conditions of assay.

Expression of results. Enzymic activity has been expressed as units of enzyme/ml.

culture. The protein content of the culture, from which each extract was derived, was known to be 50 % of the dry wt. of organism, and the amount of enzyme in units/ml. culture was derived from the specific activity of the extract (units of enzyme/mg. of extract protein). Results are plotted as units of enzyme or μm-mole of bacteriochlorophyll/ml. culture against the dry wt. organism, so that the slopes represent the differential rate of synthesis (the ratio of the rate of enzyme synthesis to the rate of increase in total mass of organism). The theoretical basis of this measurement was discussed by Monod, Pappenheimer & Cohen-Bazire (1952).

Chemicals. Stock solutions of haemin (Roche Products Ltd., Welwyn Garden City, Herts.) and protoporphyrin (L. Light and Co., Colnbrook, Bucks.) were at a concentration of 1 mM in 50 % (w/v) ethanol containing 0·05 M-$NaHCO_3$. The potassium salt of magnesium protoporphyrin was prepared from protoporphyrin methyl ester by the method of Granick (1948). Stock solutions (0·4 mM) were prepared as above. Sources of other compounds used were described previously (Lascelles, 1959).

RESULTS

The effect of incubation conditions on rate of growth of Rhodopseudomonas spheroides *and bacteriochlorophyll synthesis*

The rate of growth under the three types of standard incubation (anaerobically in the light and aerobically in the dark with high and low aeration) were similar, the mean generation time being 2·5–3 hr. (Fig. 2*a–c*). When growth ceased due to exhaustion of the carbon source the final yield of organisms in anaerobic + light cultures was almost twice that in aerobic cultures, as observed by Cohen-Bazire *et al.* (1957).

Synthesis of bacteriochlorophyll occurred anaerobically in the light, but in the dark the pigment was formed only under low aeration and reached less than one-third the concentration attained anaerobically in the light (Fig. 2). Increasing the light intensity with anaerobic cultures increased the growth rate but depressed the synthesis of bacteriochlorophyll (Fig. 2*a*), as previously shown by Cohen-Bazire *et al.* (1957).

Effect of incubation conditions on the differential rate of synthesis of δ-amino-laevulic acid synthetase and dehydrase

The rates of synthesis of both ALA synthetase and ALA dehydrase were considerably influenced by the conditions of incubation. In cultures incubated anaerobically in the light under the standard conditions synthesis of both enzymes occurred at a constant rate, the amount of enzyme activity increasing linearly with the total mass of organisms between the limits 0·3–1·0 mg. dry wt./ml. (Fig. 3*a*). Under these conditions the differential rate of synthesis of ALA synthetase varied in different experiments from 60 to 85 units/mg. dry wt. organism while that for ALA dehydrase was between 50 and 70.

In cultures growing in the dark the rate of synthesis of both enzymes varied according to the degree of aeration. Under high aeration, where no bacteriochlorophyll was formed, the rates were decreased to about one-third of that found anaerobically in the light (Fig. 3*b*). Under low aeration, where bacteriochlorophyll

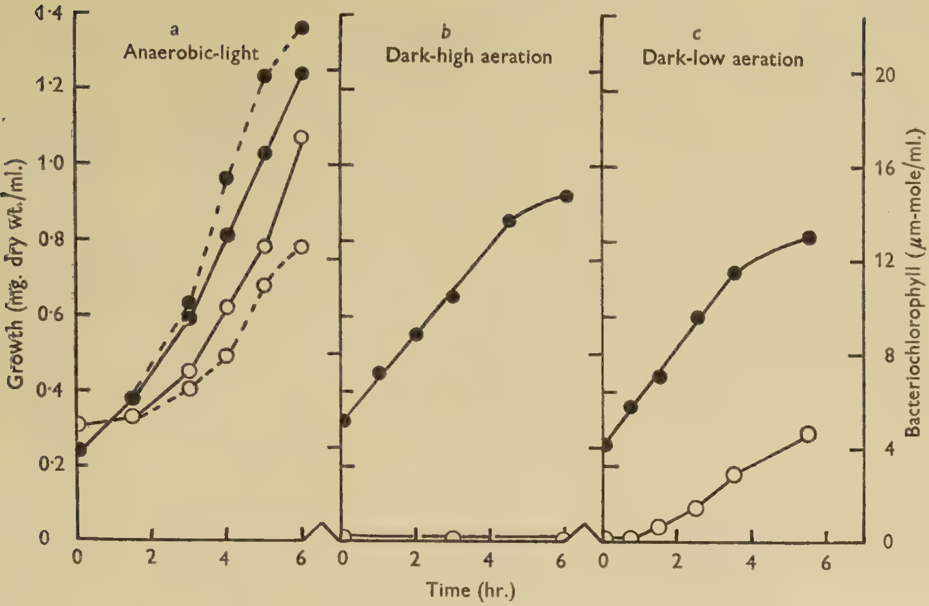

Fig. 2. Effect of incubation conditions on the rate of growth of *Rhodopseudomonas spheroides* (–●–) and bacteriochlorophyll synthesis (–○–). In (*a*) organisms harvested after anaerobic growth in light were resuspended in fresh medium and incubated anaerobically in light under standard conditions (–) or in front of a 275 W. photoflood lamp (- - -). In (*b*) and (*c*) organisms harvested after aerobic growth in dark were resuspended in fresh medium and incubated in dark under high aeration (*b*) or low aeration (*c*) as described in Methods.

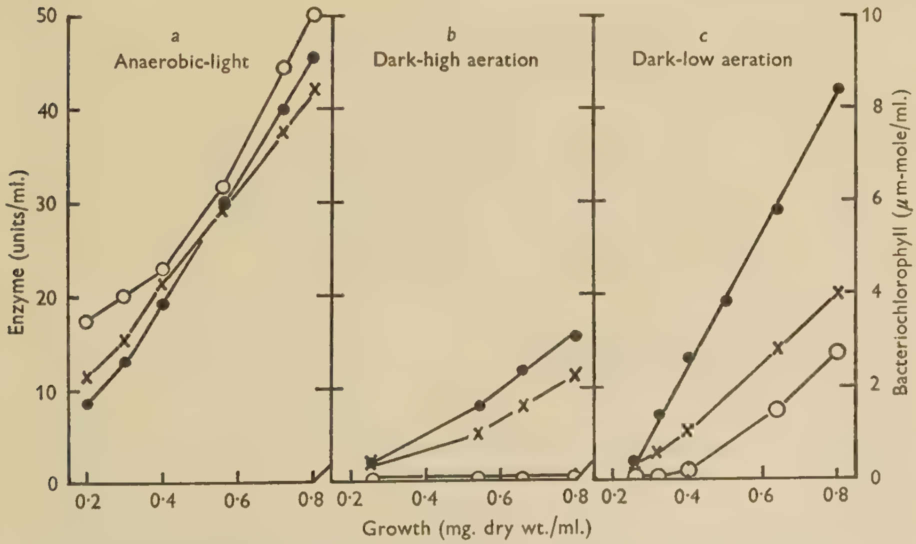

Fig. 3. Effect of incubation conditions on the synthesis of ALA synthetase (–●–), ALA dehydrase (– × –) and bacteriochlorophyll (–○–). In (*a*) cultures were harvested after anaerobic growth in light, resuspended in fresh medium and incubated anaerobically in light under standard conditions. In (*b*) and (*c*) cultures were harvested after aerobic growth in dark, resuspended in fresh medium and incubated in dark under high aeration (*b*) or low aeration (*c*) as described in Methods.

was synthesized, the rate of formation of ALA synthetase was the same as in anaerobic + light cultures, while that of ALA dehydrase was about 70 % of that found anaerobically (Fig. 3*c*). Unlike enzyme synthesis under these conditions, the rate of bacteriochlorophyll formation did not reach a steady state. Synthesis of the pigment was not detected until the culture density reached about 0·4 mg./ml. and thereafter it was formed at an increasing rate (Fig. 3*c*).

The assay for ALA synthetase depended on the presence in the organisms of succinic thiokinase. The activity of this enzyme in extracts of organisms grown under all conditions described above was constant. When assayed as described previously (Lascelles, 1959) the specific activity was about 2·1 μmole succinhydroxamate formed/hr./mg. protein, whatever the growth conditions.

Effect of light intensity. The rate of formation of ALA synthetase by cultures incubated anaerobically in the light was influenced by the light intensity in the same way as was bacteriochlorophyll synthesis. When the illumination was increased by changing the light source from the standard one (a 100 W. lamp) to a 275 W. photoflood lamp the rate of formation of ALA synthetase was decreased to about one-half of that in the control culture incubated continuously under standard conditions (Fig. 4). The rate of synthesis of ALA dehydrase was decreased to a similar extent.

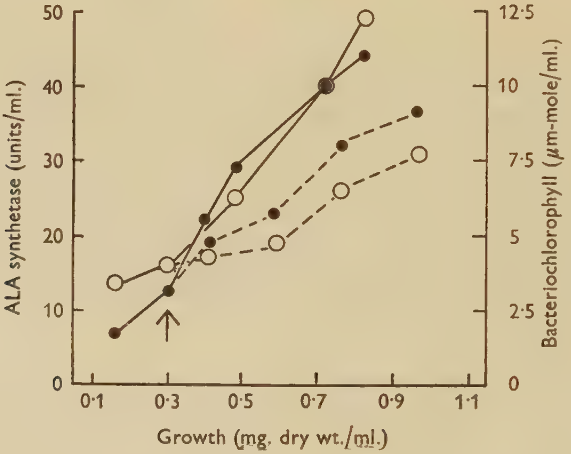

Fig. 4. Effect of light intensity on the synthesis of ALA synthetase (–●–) and bacteriochlorophyll (–○–). Organisms harvested after anaerobic growth in light were resuspended in fresh medium and incubated for 2·25 hr. anaerobically in light under standard conditions. At the point indicated by the arrow the culture was divided and one half incubated in front of a 275 W. photoflood lamp (broken lines); the other half was continuously incubated under the standard conditions (100 W. lamp).

Effect of oxygen. Cohen-Bazire *et al.* (1957) found that synthesis of bacteriochlorophyll by cultures of *Rhodopseudomonas spheroides* growing in the light ceased abruptly when the gas phase of $N_2 + CO_2$ was changed to one of air + CO_2. Similar experiments under the present conditions showed that formation of both ALA synthetase and dehydrase was repressed by replacement of N_2 by air in the same way as bacteriochlorophyll synthesis (Fig. 5*a*, *b*); growth continued at the same rate when the gas phase was changed. Synthesis of both enzymes and of bacteriochlorophyll was resumed without a detectable lag when the gas phase was changed back to 5 % (v/v) CO_2 in N_2 (Fig. 5*a*, *b*). Indeed, the rates of synthesis of ALA synthetase and bacteriochlorophyll were increased after the period of aerobic incubation, so that the values quickly reached those found in control cultures incubated continuously in N_2+CO_2 (Fig. 5*a*, *b*).

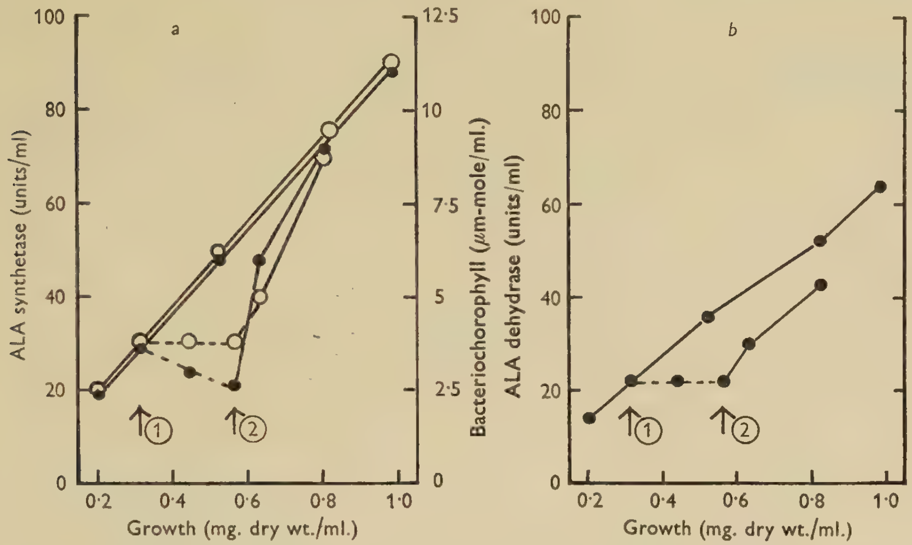

Fig. 5. Effect of oxygen on (*a*) the synthesis of ALA synthetase (–●–) and bacteriochlorophyll (–○–) and (*b*) on ALA dehydrase (–●–). Organisms, harvested after anaerobic growth in light, were resuspended in fresh medium and incubated for 2 hr. in light under 5 % (v/v) CO_2 in N_2. At the point indicated by the arrow (1) the culture was divided into two parts. One part was aerated with 5 % (v/v) CO_2 in air for a further 1·75 hr. (broken lines) and at the point shown by arrow (2) the gas phase was changed back to N_2+CO_2. The other part of the culture (full lines) was kept continuously under N_2+CO_2. All cultures were incubated with continuous illumination.

The repressing action of δ-aminolaevulic acid and protoporphyrin derivatives

Formation of ALA synthetase and ALA dehydrase by cultures growing anaerobically in the light was inhibited by addition of ALA at concentrations (0·1–0·5 mM) which affected neither growth nor bacteriochlorophyll synthesis (Fig. 6, Table 1). Addition of ALA resulted initially in an actual decrease of ALA synthetase in the culture (Fig. 6); this was consistently observed. One possibility was that the organisms were forming from ALA a substance which inhibited ALA synthetase when assayed in the cell-free extracts. However, the ALA synthetase activity of extracts from organisms grown without ALA was not inhibited by addition of extracts prepared from cultures grown with ALA.

The inhibition of enzyme formation may not have been due to ALA itself, but to some metabolic product, possibly an intermediate in the synthesis of haem compounds or bacteriochlorophyll. Suspensions of *Rhodopseudomonas spheroides*, incubated anaerobically in the light with iron salts, convert ALA to protoporphyrin and iron protoporphyrin (Lascelles, 1956*a*, *b*) and the effect of these compounds on enzyme synthesis was therefore examined (Table 1). Low concentrations of iron protoporphyrin (added as haemin) inhibited the synthesis of both ALA synthetase and dehydrase but similar concentrations of protoporphyrin were ineffective. Also, magnesium protoporphyrin, a possible intermediate between ALA and bacteriochlorophyll, was inactive at concentrations up to 0·005 mM.; higher concentrations were not tested because of the insolubility of this compound.

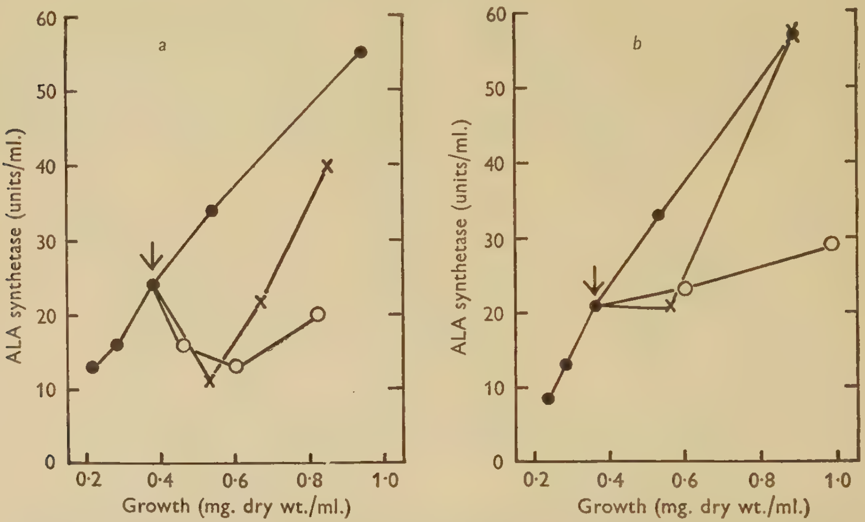

Fig. 6. Effect of ALA (*a*) and haemin (*b*) on the synthesis of ALA synthetase. Organisms, grown anaerobically in light, were harvested and suspended in fresh medium. In both experiments the cultures were incubated anaerobically in light for 2 hr. before dividing into equal portions and adding ALA or haemin (shown by the arrow). In (*a*) ALA was added to a final concentration of 0·1 mM (— × —) and 0·5 mM (—○—); no addition was made to the control culture (—●—). In (*b*), haemin was added to give a final concentration of 0·003 mM (— × —) and 0·01 mM (—○—); no addition was made to the control (—●—).

Unlike the effect of ALA, addition of haemin to cultures did not result in an apparent decrease in the amount of ALA synthetase (Fig. 6*b*). The action of the two compounds was similar in that enzyme synthesis was gradually restored to the normal rate as growth proceeded after their addition. They may have been diluted out or converted to products which did not affect formation of ALA synthetase.

Effect of biotin

Rhodopseudomonas spheroides requires biotin for growth (Hutner, 1950) and it was observed previously that suspensions of biotin-deficient organisms did not synthesize porphyrins from glycine and α-oxoglutarate though their ability to form them from ALA was unimpaired (Lascelles, 1956*a*). This suggested that biotin may be required either for the formation or for the normal functioning of ALA synthetase. The observation that the activity of ALA synthetase was decreased in extracts of biotin-deficient organisms supports this (personal communication from Professor A. Neuberger and Mr G. Tait).

The rate of formation of ALA synthetase by cultures growing anaerobically in the light was apparently influenced by the concentration of biotin in the medium. In these experiments organisms were harvested after anaerobic growth in the light in the standard medium modified by addition of biotin to 0·2 μg./l., this being one-fiftieth the usual concentration. On transfer to fresh medium the organisms formed ALA synthetase at a differential rate which depended on the biotin concentration. In the control culture containing optimal biotin (10 μg./l.) the differential rate was 83, but in cultures containing one-tenth and one-fiftieth this concentration the differential rates were decreased to 56 and 34, respectively (Fig. 7*a*). Synthesis of

Table 1. *Effect of δ-aminolaevulic acid and tetrapyrroles on synthesis of enzymes by cultures of* Rhodopseudomonas spheroides *growing anaerobically in the light*

Organisms harvested after anaerobic growth in light were resuspended in fresh medium to a density equiv. 0·28 mg. dry wt./ml. Medical flat bottles (2 oz.) were filled to the neck with 60 ml. portions of the suspension, additions made as shown and the bottles sealed with a rubber bung. The cultures were incubated for 4 hr. at 34° in front of a bank of 60 W. tungsten lamps. They were then prepared as usual for assay of enzyme activity.

Initial activities of the cultures before incubation were: ALA synthetase, 11 units/ml.; ALA dehydrase, 17 units/ml.; initial bacteriochlorophyll = 4 μm-mole/ml.

	Increase in				Differential rate of enzyme synthesis	
			Enzymes (units/ml.)			
Compound added	Suspension density as dry wt. (mg./ml.)	Bacterio-chlorophyll (μm-mole/ml.)	Synthetase	Dehydrase	Synthetase	Dehydrase
Nil	0·40	7·1	34	25	85	62·5
ALA 0·5 mM	0·38	6·8	8	13	21	34
Haemin 0·01 mM	0·40	6·7	11	14	28	35
Protoporphyrin 0·01 mM	0·40	7·0	33	24	83	60
Mg-protoporphyrin 0·005 mM	0·44	7·8	35	27	80	61

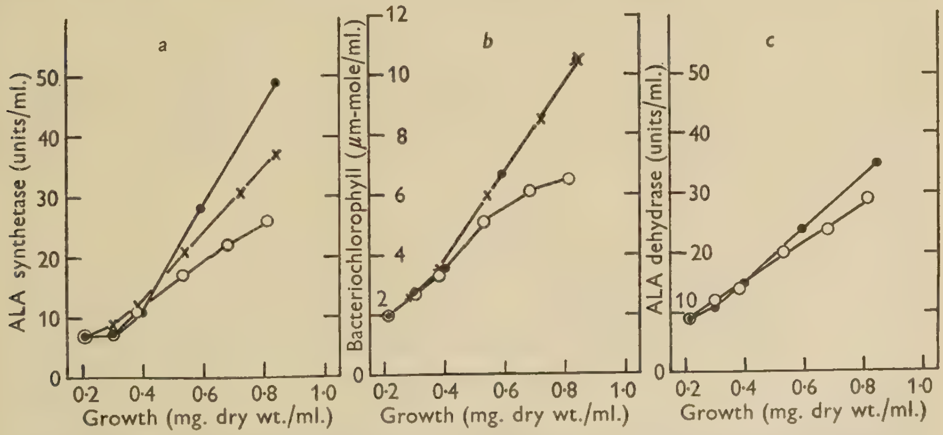

Fig. 7. Effect of biotin concentration on the synthesis of ALA synthetase (*a*), bacteriochlorophyll (*b*) and ALA dehydrase (*c*). Organisms were harvested after anaerobic growth in light in the standard medium except that the biotin concentration was decreased to 0·2 μg./l.; they were resuspended in biotin-free medium and portions incubated anaerobically in light with addition of biotin as follows (μg./l.): 0·2 (–○–); 1 (–×–) and 10 (–●–). Samples were removed and prepared for assay as usual.

bacteriochlorophyll was also decreased by biotin deficiency though to a smaller extent than was ALA synthetase (Fig. 7*b*). In contrast, the differential rate of synthesis of ALA dehydrase was not affected (Fig. 7*c*). The mean generation time of the cultures containing 10, 1 and 0·2 μg. biotin/l. were, respectively, 2·8, 3·5 and 4·4 hr. Addition of biotin to extracts of deficient organisms did not increase their ALA synthetase activity.

DISCUSSION

It is clearly an advantage for *Rhodopseudomonas spheroides* and related organisms to be able to regulate the amount of bacteriochlorophyll synthesized to conform to the requirements imposed by the environment. Bacteriochlorophyll synthesis results in the utilization of glycine and succinyl CoA, which are intermediates in other essential metabolic reactions; a control mechanism which operates at the first stage in the synthesis, involving condensation of these compounds (i.e. ALA synthetase), might therefore be important to the organism. Such a mechanism would have to be delicately balanced since the bacteria must be able to synthesize some ALA under all conditions of growth in order to make haematin compounds, such as cytochromes, which are formed anaerobically in the light and aerobically in the dark.

The amount of ALA synthetase might be important in regulating the amount of glycine and succinyl CoA diverted towards tetrapyrrole synthesis; the present work has shown that synthesis of this enzyme is influenced by the conditions of growth in the same way as that of bacteriochlorophyll. Under highly aerobic conditions the differential rate of synthesis is about one-third of that in cultures growing anaerobically in the light. Also, synthesis of the enzyme is repressed by oxygen in cultures growing under continuous illumination. The light intensity, which influences bacteriochlorophyll formation under anaerobic conditions, also affects the synthesis of ALA synthetase, the differential rate being decreased by increasing the light intensity. Formation of ALA dehydrase is affected by the various conditions in the same way as its ALA synthetase. This is in accord with observations on other metabolic pathways in which formation of a number of enzymes in a biosynthetic sequence is repressed simultaneously by the same agent (see, for instance, Ames & Garry, 1959). The amounts of enzymes concerned in later stages of tetrapyrrole synthesis are probably also influenced by the environment. The enzyme systems in *Rhodopseudomonas spheroides* responsible for conversion of porphobilinogen to uro- and coproporphyrinogens (see Fig. 1), which have been studied recently (Heath & Hoare, 1959; Hoare & Heath, 1959) are less active in extracts from aerobic organisms than in those from photosynthetically grown organisms (personal communication from Dr D. Hoare).

The present work has given no indication of the precise mechanism by which oxygen exerts its repressing effect on enzyme synthesis. The hypothesis of Cohen-Bazire *et al.* (1957) that pigment synthesis is regulated by the concentration of a component in the electron transport chain seems, from the present data, to apply equally well to the synthesis of the enzymes. The repressing effect of oxygen on the formation of ALA synthetase and dehydrase cannot fully account for its action on bacteriochlorophyll synthesis. If this were so pigment formation would not cease abruptly on the introduction of oxygen but would proceed at a decreasing differential rate as growth continued and the enzymes in the culture were diluted out. Oxygen may possibly inhibit the action of one or more of the enzymes concerned in bacteriochlorophyll synthesis. Such an affect has been observed with chick red cells where conversion of glycine to porphyrins and haem is inhibited by high tensions of oxygen (Falk, Porra, Brown, Moss & Larminie, 1959). Another possibility is that oxygen acts indirectly by causing the accumulation of a substance which inhibits the action of a key enzyme.

It is difficult to assess the physiological significance of the repressing effect shown with δ-aminolaevulic acid (ALA). In the many examples of enzyme repression now known to operate in a variety of biosynthetic pathways the end product of the reaction sequence (e.g. an amino acid) prevents enzyme synthesis (see, for instance, Vogel, 1957). By analogy, ALA itself may not exert the repressing effect but may act by serving as a precursor of an active tetrapyrrole; it is possibly significant that haemin also is an active repressor of the formation of ALA synthetase and dehydrase. More definite information must wait until synthesis of the enzymes has been obtained in cell-free systems where there is no problem of impermeability to products of ALA metabolism and until more is known of the pathways leading from protoporphyrin to bacteriochlorophyll and to haem proteins.

The observed effects of biotin deficiency might be interpreted in two ways. Biotin, probably in some combined form, may be a cofactor of ALA synthetase or it may be needed for the synthesis of the enzyme. The fact that addition of biotin to deficient extracts had no effect is not necessarily significant since the extracts may have been unable to convert it to its coenzyme form. The nature of the biotin effect cannot be established until the enzyme has been purified and its mechanism of action established. Recent work has shown biotin to be a cofactor in several enzymes concerned in the metabolism of fatty acids; all the reactions involve transfer of CO_2 (Lynen, Knappe, Lorch, Jütting & Ringelmann, 1959). It may be significant that the action of ALA synthetase results in the liberation of CO_2 from the carboxyl group of glycine (Kikuchi *et al.* 1959).

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The Formation of Ribulose 1:5-Diphosphate Carboxylase by Growing Cultures of Athiorhodaceae

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SUMMARY

Ribulose 1:5-diphosphate carboxylase, which catalyses the conversion of ribulose 1:5-diphosphate and CO_2 to 3-phosphoglyceric acid, is a key enzyme in the reductive pentose cycle. It was formed only in traces by *Rhodopseudomonas spheroides* growing in the dark under conditions of high or low aeration but organisms grown photosynthetically were rich in the enzyme. The activity of some other enzymes of the cycle, phosphoriboisomerase, phosphoglyceric acid kinase and triosephosphate dehydrogenase (diphosphopyridine nucleotide-linked) did not vary significantly with the growth conditions. The differential rate of synthesis (increase in enzyme/increase in culture density) of the carboxylase was studied in cultures of *R. spheroides* growing exponentially under various conditions. It was influenced by the light intensity, being decreased when this was increased. Oxygen completely repressed formation of the enzyme even under continuous illumination; this effect was annulled by restoration of anaerobic conditions. Dark-grown organisms formed the enzyme at a high differential rate immediately on transfer to anaerobic + light conditions, provided that they contained a minimal amount of bacteriochlorophyll. Biotin deficiency did not affect the synthesis of the carboxylase. The conditions which promoted carboxylase formation were in many respects similar to those favourable to synthesis of enzymes required for the early stages of bacteriochlorophyll formation. The connexion of these observations with the regulation of pigment synthesis and with the possible physiological role of the enzyme in the Athiorhodaceae is discussed.

INTRODUCTION

The enzyme (RuDP carboxylase) catalysing the formation of 3-phosphoglyceric acid from ribulose 1:5-diphosphate (RuDP) and CO_2 is a key one in the reductive pentose cycle (Bassham & Calvin, 1957) and was first demonstrated in preparations from algae (Quayle, Fuller, Benson & Calvin, 1954; Weissbach, Smyrniotis & Horecker, 1954). Its presence has since been shown either directly or indirectly in many autotrophic bacteria (Trudinger, 1956; Aubert, Milhaud & Millet, 1957; Vishniac & Santer, 1957; Bergmann, Towne & Burris, 1958; Kornberg, Collins & Bigley, 1960) as well as in *Pseudomonas oxalaticus* growing on formate (Quayle & Keech, 1959*a*, *b*). The operation of the reductive pentose cycle in photosynthetic bacteria was suggested originally by observations that 3-phosphoglyceric acid appeared as an early product of $^{14}CO_2$ fixation by *Rhodospirillum rubrum* and

Rhodopseudomonas capsulatus; this occurred when organisms harvested after photosynthetic growth were incubated anaerobically in light or aerobically in dark in the presence of oxidizable substrates (Glover, Kamen & van Genderen, 1952; Stoppani, Fuller & Calvin, 1955). The presence of RuDP carboxylase has since been directly demonstrated in extracts of *Rhodospirillum rubrum* and *Chromatium* sp. (Fuller & Gibbs, 1959).

RuDP carboxylase is formed by chemosynthetic organisms, which use either hydrogen or organic substrates, only under conditions of autotrophic growth (Vishniac & Santer, 1957; Kornberg *et al.* 1960). It is also formed adaptively by *Pseudomonas oxalaticus* in response to formate, which is assimilated via the reductive pentose pathway, but oxalate (which is assimilated by a different route) does not induce the enzyme (Quayle & Keech, 1959*a*, *b*; 1960). The carboxylase is also adaptive in algae and is found only in organisms grown in the light with CO_2 as carbon source (Fuller & Gibbs, 1959).

It seemed probable that the formation of RuDP carboxylase by the Athiorhodaceae might also be influenced by the environment; many of these organisms are capable of aerobic growth in the dark on the same organic substrates that are utilized for photosynthetic development (van Niel, 1944). In the present work a study has been made of the effect of various factors on RuDP carboxylase formation in exponentially growing cultures of *Rhodopseudomonas spheroides*; conditions for synthesis of the enzyme have been found to be similar to those shown previously to favour formation of bacteriochlorophyll and enzymes concerned in the early stages of tetrapyrrole synthesis (Lascelles, 1960).

METHODS

Organisms. Rhodopseudomonas spheroides (NCIB no. 8253) and *R. palustris* (strain 2.1.7 from the collection of Professor C. B. van Niel) were used. Stock cultures of both were maintained as described previously (Lascelles, 1956).

Media. Unless stated otherwise all experiments with *Rhodopseudomonas spheroides* were made with cultures grown in a chemically defined medium containing malate and glutamate supplemented with 0·01 mM-iron citrate (Lascelles, 1959). *p*-Aminobenzoate (0·001 mM) was added to this medium for growth of *R. palustris*. In one experiment, shown in Table 1, *R. spheroides* was grown on a medium containing sodium butyrate (0·01 M) and sodium bicarbonate (0·006 M) in place of malate and glutamate.

Growth of cultures. The methods used to obtain exponentially growing cultures and the standard conditions for anaerobic incubation in the light and for aerobic incubation in the dark under conditions of high and low aeration are described in the previous paper (Lascelles, 1960); growth was also assessed in the way there indicated.

Preparation of cell-free extracts. Samples were taken from cultures and extracts prepared from the harvested organisms as described previously (Lascelles, 1960).

Estimation of bacteriochlorophyll and protein. These were determined by the methods described by Lascelles (1959).

Assays of enzymic activity. RuDP carboxylase was assayed by a method based on that described by Racker (1957) in which the reaction product, 3-phosphoglyceric acid, is estimated spectrophotometrically in the presence of reduced diphosphopyridine nucleotide (DPNH), adenosine triphosphate (ATP), 3-phosphoglyceric acid kinase, and triosephosphate dehydrogenase. The assay mixture contained: extract of organisms (0·1–0·5 mg. protein); 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·8), 55 μmole; $MgCl_2$, 5 μmole; glutathione (freshly neutralized), 6 μmole; $NaHCO_3$, 40 μmole; RuDP (potassium salt), 0·5 μmole; water to 1·0 ml. The mixtures were incubated for 10 min. at 30° and the reaction stopped by adding 0·02 ml. N-HCl and heating at 100° for 2 min. After centrifugation, the 3-phosphoglyceric acid formed was estimated in samples of the supernatant fluid. For this purpose micro-cuvettes (1 cm. light path, 0·4 ml. capacity) were filled as follows: sample, 0·2 ml.; tris buffer (pH 7·8), 25 μmole; $MgCl_2$, 2·5 μmole; L-cystein (freshly neutralized), 3 μmole; crystalline triosephosphate dehydrogenase, 25 μg.; ATP, 0·3 μmole; DPNH, 0·056 μmole; water to final volume 0·4 ml. After taking the initial reading at 340 mμ (against the control cuvette containing the complete reaction mixture without DPNH) the reaction was started by addition of 3-phosphoglyceric acid kinase (2·5 μg.). Readings were taken until there was no further decline in extinction at 340 mμ (5–10 min.) and the amount of 3-phosphoglyceric acid in the sample calculated from the amount of DPNH oxidized; 10 μm-mole of 3-phosphoglyceric acid caused a decline in extinction of 0·155. Controls without bicarbonate were included in each assay and the 3-phosphoglyceric acid formed was deducted from the experimental value; the amount was small and did not exceed 15 μm-mole/mg. of protein of the extract. One unit of enzyme is defined as the amount which catalyses the formation of 1 μm-mole of 3-phosphoglyceric acid under the standard conditions of assay.

Phosphoriboisomerase, which catalyses the conversion of ribose-5-phosphate to ribulose-5-phosphate, was assayed by the method described by Horecker, Hurwitz & Weissbach (1958) except that incubation was for 10 min. at 30°. One unit of enzyme is defined as the amount catalysing the formation of 1 μm-mole ketopentose under the standard conditions.

Triosephosphate dehydrogenase and 3-phosphoglyceric acid kinase were assayed spectrophotometrically at 340 mμ by following the rate of oxidation of DPNH in the presence of 3-phosphoglyceric acid, ATP and excess of one or other of these enzymes (Bücher, 1947). For assay of the dehydrogenase, cuvettes (1 cm. light path, 4 ml. total capacity) were filled as follows: extract of organisms (0·05–0·1 mg. protein); tris buffer (pH 8·2), 40 μmole; L-cystein (freshly neutralized), 12 μmole; ATP, 2 μmole; DPNH, 0·3 μmole (except in the blank); 3-phosphoglyceric acid kinase, 5 μg.; water to final volume 3·1 ml. The reaction was started by adding 10 μmole 3-phosphoglyceric acid and readings at 340 mμ were taken at 1 min. intervals to determine the rate of DPNH oxidation. 3-Phosphoglyceric acid kinase was estimated in the same way except that the assay mixture contained triosephosphate dehydrogenase (10 μg.) in place of the kinase. One unit of either enzyme is the amount which catalyses a decline in extinction of 0·10 in 2 min. under the standard conditions.

δ-Aminolaevulic acid synthetase (ALA synthetase) was assayed as described previously (Lascelles, 1960).

All spectrophotometric determinations were made with the Unicam spectrophotometer, Model SP 500.

Expression of results. As in the previous paper (Lascelles, 1960) most of the results are presented as the differential rate of enzyme synthesis, i.e. the ratio of increase in enzyme to increase in culture density (Monod, Pappenheimer & Cohen-Bazire, 1952).

Materials. Ribulose 1:5-diphosphate (potassium salt) was prepared from ribose-5-phosphate and ATP by the action of phosphoriboisomerase and phosphoribulokinase; the methods of preparation of the phosphate ester and the enzymes (from spinach) were as described by Horecker *et al.* (1958). The potassium salts of ribose-5-phosphate and 3-phosphoglyceric acid were made from the barium salts (British Drug Houses Ltd., Poole, Dorset) by treatment with Dowex-50 (H^+ form) and neutralization with N-KOH. Adenosine triphosphate, DPNH and triphosphopyridine nucleotide (TPNH) were from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Crystalline triosephosphate dehydrogenase and 3-phosphoglyceric acid kinase were purchased from Boehringer & Soehne (Mannheim, Germany).

RESULTS

Effect of light on the production of various enzymes

Enzymes in Rhodopseudomonas spheroides. Extracts of organisms harvested after anaerobic growth in light were rich in RuDP carboxylase, but only traces of this enzyme were found in those from cultures which had been grown in the dark under high aeration (Table 1). There was little difference in the amount of RuDP carboxylase in organisms grown in the dark under either low or high aeration; this is in contrast to the ALA synthetase, the activity of which is high in organisms grown under low aeration (Table 1).

Table 1. *Activity of enzymes in extracts of* Rhodopseudomonas spheroides *and* R. palustris *grown under various conditions*

Extracts were prepared from organisms harvested during the logarithmic phase of growth under the conditions described in the Methods. The results shown are the mean values obtained with at least two extracts of each type.

Organism	Medium	Enzyme	Activity in extracts from organisms grown (units enzyme/mg. protein)		
			Dark		Anaerobic + light
			Low aeration	High aeration	
Rhodopseudomonas spheroides	Malate + glutamate	RuDP carboxylase	15	10	300
		ALA synthetase	122	16	150
		Phosphoriboisomerase	nt	435	520
		3-Phosphoglycerate kinase	nt	0·11	0·12
		Triosephosphate dehydrogenase	nt	0·76	0·71
	Butyrate + $NaHCO_3$	RuDP carboxylase	nt	< 10	342
R. palustris	Malate + glutamate	RuDP carboxylase	nt	82	273

nt = not tested.

Rhodopseudomonas spheroides, like other members of the Athiorhodoaceae, requires added bicarbonate for growth on fatty acids even under aerobic conditions (van Niel, 1941) and the activity of RuDP carboxylase was therefore examined in extracts of organisms grown on these substrates. The amount of the enzyme was, however, just as low in extracts of bacteria grown aerobically on media containing butyrate + bicarbonate in place of malate + glutamate; extracts from photosynthetically-grown cultures on either medium had similar activity (Table 1). Similar results were obtained with extracts from cultures on acetate + bicarbonate.

The growth conditions did not significantly affect the activity of enzymes involved at other stages in the reductive pentose cycle. Phosphoriboisomerase, 3-phosphoglycerate kinase and triosephosphate dehydrogenase had similar activities in extracts of organisms grown either in the light or in the dark (Table 1). The triosephosphate dehydrogenase in organisms grown aerobically or photosynthetically reacted only with DPNH and there was no sign of activity with TPNH. This is in contrast to green plants which contain both di- and triphosphopyridine nucleotide-linked triosephosphate dehydrogenases (Gibbs, 1952; Arnon, Rosenberg & Whatley, 1954).

RuDP carboxylase in Rhodopseudomonas palustris. This organism, like *R. spheroides*, grows both aerobically and photosynthetically, forming bacteriochlorophyll under the latter conditions. Appreciable RuDP carboxylase was found in extracts of aerobically-grown organisms, but it was only *c.* 30 % of that in extracts from bacteria grown anaerobically in light (Table 1).

The differential rate of synthesis of ribulose diphosphate carboxylase by Rhodopseudomonas spheroides

Effect of light intensity. Cultures of *Rhodopseudomonas spheroides* growing anaerobically in light under the standard conditions formed RuDP carboxylase at a fairly constant differential rate, which varied from 140 to 200 units/mg. dry wt. organism in different experiments. This was decreased by a factor of two or more when the illumination was increased by changing the light source from a 100 W. lamp to a 275 W. photoflood lamp (Fig. 1). Such a change also results in decreases in the differential rate of synthesis of both bacteriochlorophyll and ALA synthetase (Lascelles, 1960).

Effect of O_2. Synthesis of RuDP carboxylase ceased abruptly when cultures growing anaerobically in light were transferred to the dark and the incubation continued aerobically (Fig. 2). Cessation of synthesis under these conditions might have been due to the absence of light or to the presence of oxygen; to determine this, formation of the carboxylase was examined in cultures growing under continuous illumination but with changes in the gas phase. Synthesis of the enzyme stopped completely when the gas phase of 5 % (v/v) CO_2 in N_2 was replaced by one of 5 % (v/v) CO_2 in air, though growth continued at the same rate (Fig. 3). This change was reversible, enzyme synthesis being resumed at the same differential rate when the gas phase was changed back to 5 % (v/v) CO_2 in N_2 (Fig. 3). The effect of O_2 on the formation of the carboxylase enzyme is therefore similar to its action on synthesis of bacteriochlorophyll and ALA synthetase (Lascelles, 1960).

Enzyme synthesis by Rhodopseudomonas spheroides *adapting from aerobicto photosynthetic conditions.* The formation of RuDP carboxylase and bacteriochlorophyll was examined in cultures of *R. spheroides* which were adapting from aerobic growth in the dark to anaerobic growth in light. When organisms harvested after growth in the dark under high aeration were resuspended in fresh medium and incubated anaerobically in light, growth occurred after a lag of about 14 hr. (Fig. 4*a*). Such organisms initially contain only traces of bacteriochlorophyll and growth cannot occur until sufficient pigment has been made for the bacteria to obtain energy from the light. The development of RuDP carboxylase in such cultures occurred simultaneously with the formation of bacteriochlorophyll (Fig. 4*a*).

A different situation was found when aerobically grown organisms were subjected to a short period of incubation under low aeration in the dark before transferring to anaerobic conditions in light. The preliminary incubation resulted in a 16-fold increase in the concentration of bacteriochlorophyll in the organisms (from 0·18 to 2·9 μm-mole/mg. dry wt.) but there was no detectable increase in the RuDP carboxylase. On changing to anaerobic conditions in light there was a lag of 1–2 hr. before the rate of growth and bacteriochlorophyll formation became maximal, but synthesis of RuDP carboxylase occurred without a detectable lag (Fig. 4*b*). Thus, initially the enzyme was formed at a greater rate than the total protein of the organisms, as shown by the high differential rate; this decreased to the usual value when the culture entered the exponential phase of growth (Fig. 5). Bacteriochlorophyll was formed at a constant differential rate throughout (Fig. 5).

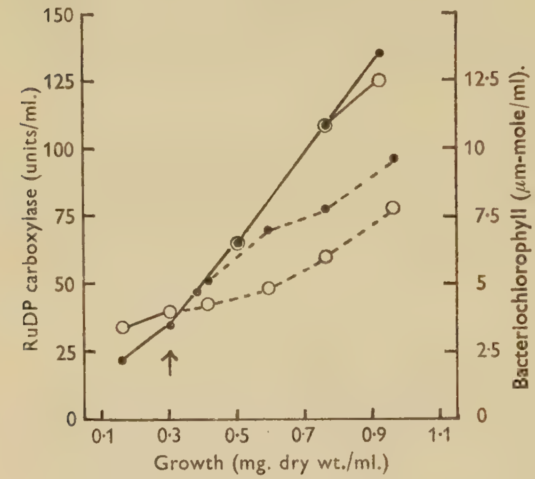

Fig. 1

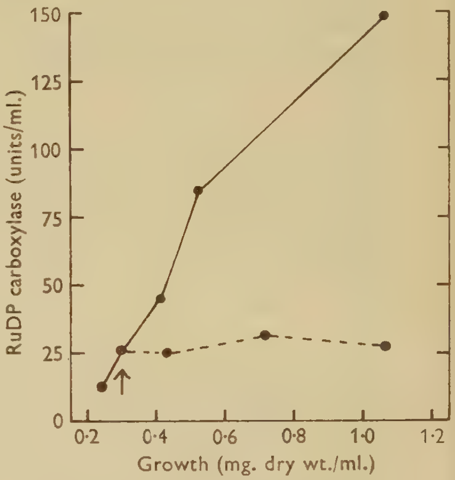

Fig. 2

Fig. 1. Effect of light intensity on synthesis of RuDP carboxylase (—●—) and bacteriochlorophyll (—○—) by *Rhodopseudomonas spheroides*. Organisms harvested after anaerobic growth in light were resuspended in fresh medium and incubated for 2·25 hr. anaerobically in light under standard conditions. At the point shown by the arrow the culture was divided and one half incubated in front of a 275 W. photoflood lamp (broken lines); the other half was continuously incubated under the standard conditions (100 W. lamp).

Fig. 2. Effect of aerobic incubation in the dark on synthesis of RuDP carboxylase. Organisms harvested after anaerobic growth in the light were resuspended in fresh medium and incubated for one hour anaerobically in the light under standard conditions. At the stage shown by the arrow the culture was divided, one half being further incubated in the dark under high aeration (broken lines), the other half (full lines) being continuously incubated anaerobically in the light.

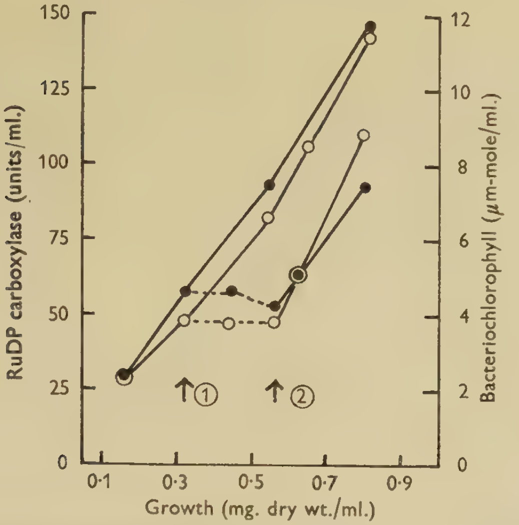

Fig. 3. Effect of oxygen on synthesis of RuDP carboxylase (—●—) and bacteriochlorophyll (—○—) by illuminated cultures. Organisms were harvested after anaerobic growth in the light, resuspended in fresh medium and incubated for 2 hr. anaerobically in the light under standard conditions. At the point shown by arrow (1) the culture was divided. One part was aerated with air-CO_2 (95:5) for a further 1·75 hr. (broken lines) and at the point shown by arrow (2) the gas phase was changed back to N_2–CO_2. The other part of the culture (full lines) was kept continuously under N_2–CO_2. All cultures were incubated with continuous illumination.

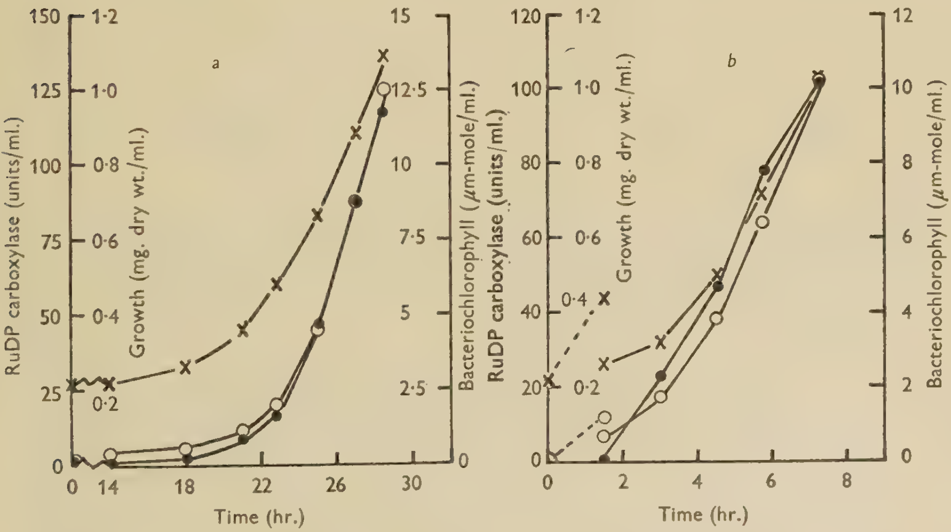

Fig. 4*a*, *b*. The rates of growth (— × —) and synthesis of RuDP carboxylase (—●—) and of bacteriochlorophyll (—○—) in cultures transferred from aerobic-dark to anaerobic-light conditions. Organisms were harvested after growth in the dark under high aeration and resuspended in fresh medium. One portion (*a*) was transferred immediately to anaerobic conditions in the light. The other portion (*b*) was incubated first for 90 min. in the dark under low aeration (broken lines), the bacteria harvested and resuspended in fresh medium and then incubated anaerobically in the light (full lines).

The effect of biotin deficiency in Rhodopseudomonas spheroides

Since biotin is known to be involved in some enzymes concerned with the transfer of CO_2 (Lynen, Knappe, Lorch, Jütting & Ringelmann, 1959) the effect of biotin deficiency on the activity of RuDP carboxylase was examined. The activity of this enzyme was, however, the same in extracts from biotin-deficient organisms (grown with one-fiftieth the usual concentration of biotin) as in those from normal organisms (Table 2). The ALA synthetase activity in the deficient extracts was only *c.* 30 % of the normal value (Table 2).

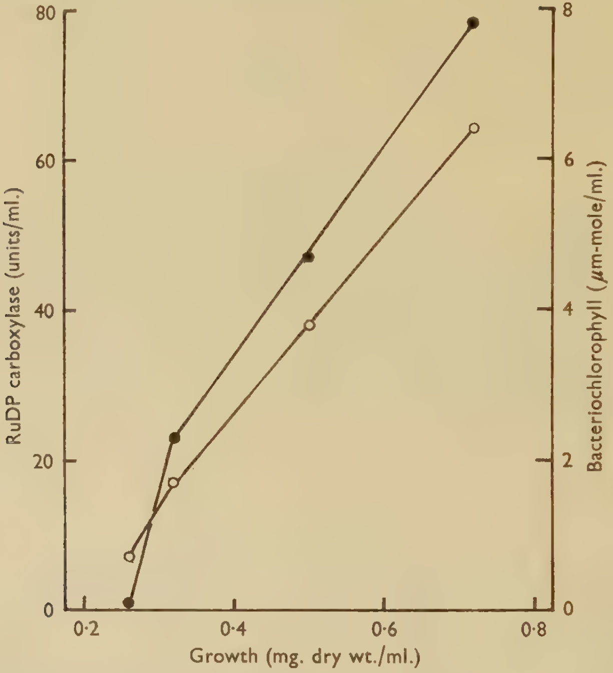

Fig. 5. The differential rate of synthesis of RuDP carboxylase (—●—) and bacteriochlorophyll (—○—) on transfer of 'pretreated' cultures to anaerobic conditions in the light. The experiment is that described in Fig. 4*b* after the cultures had been incubated for 90 min. in the dark under low aeration.

Table 2. *Effect of biotin deficiency on the activity of RuDP carboxylase and ALA synthetase in* Rhodopseudomonas spheroides

Extracts were prepared from *R. spheroides* harvested after anaerobic growth in light from cultures containing 0·2 or 10 μg./l. of biotin (deficient and normal extracts, respectively). The activities of RuDP carboxylase and ALA synthetase were assayed by the standard procedures.

	Activity of	
Extract	RuDP carboxylase (units enzyme/mg. protein)	ALA synthetase (units enzyme/mg. protein)
Deficient	320	44
Normal	340	140

DISCUSSION

The function of RuDP carboxylase in autotrophs and in *Pseudomonas oxalaticus* (on formate) is clear: it is essential for conversion of CO_2 to cell substance by the reductive pentose cycle. Its role in the Athiorhodaceae, which require organic carbon for growth, is less obvious; experiments with $^{14}CO_2$ have shown that its contribution to total cell carbon is relatively slight, the major proportion being derived from the organic substrate (Cutinelli, Ehrensvärd, Reio, Saluste & Stjernholm, 1951; Ormerod, 1956; Stanier, Doudoroff, Kunisawa & Contopoulou, 1959). Yet CO_2 plays an essential part in their metabolism and must be added to cultures or washed suspensions for utilization of reduced substrates such as fatty acids (Gaffron, 1933, 1935; van Niel, 1941; Stanier *et al.* 1959). The isotope experiments have shown that it is fixed primarily into 3-phosphoglyceric acid, at least by washed suspensions of photosynthetically-grown organisms (Glover *et al.* 1952; Stoppani *et al.* 1955); this suggests that the carboxylase does function normally in these bacteria. It is possible that its major physiological role in the Athiorhodaceae is to render CO_2 available in a form which can act as a H-acceptor under anaerobic conditions. Thus, reduction of the product of RuDP carboxylase action, 3-phosphoglyceric acid, to triose phosphate may be an important outlet for the DPNH generated by oxidation of the organic substrate. This could provide an explanation (albeit teleological) for the repressing effect of oxygen, an alternative H-acceptor which abolishes the need for CO_2 as ultimate oxidant.

The fact that the RuDP carboxylase is an adaptive enzyme in *Rhodopseudomonas spheroides*, being formed only by cultures growing anaerobically in light, supports the suggestion that the enzyme has an important function under these conditions. Enzyme synthesis occurs immediately on transfer of organisms from aerobic + dark to anaerobic + light conditions, provided that the organisms contain adequate amounts of bacteriochlorophyll. The intracellular concentration of bacteriochlorophyll does not have to be high to permit synthesis of RuDP carboxylase. In the experiment shown in Figs. 4 and 5 the initial amount of pigment (2·9 μm-mole/mg. dry wt. organism) was only about one-fifth of the maximum amount in organisms growing anaerobically in light under standard conditions; yet, enzyme synthesis began immediately at a high differential rate on exposure to anaerobic + light conditions. Similar results have been obtained with organisms containing initially as little as 1 μm-mole of bacteriochlorophyll/mg. dry wt. The rapid synthesis of the enzyme, which occurs provided that the organisms have sufficient pigment to utilize light energy, is similar to observations with *Pseudomonas oxalaticus*; synthesis of the enzyme occurs before the organism begins to grow on transfer of cultures from oxalate to formate, the energy for its synthesis being provided by oxidation of formate (Quayle & Keech, 1960).

The conditions which promote synthesis of the RuDP carboxylase are similar in several respects to those which favour bacteriochlorophyll formation. Thus, the differential rate of synthesis of the enzyme and pigment is decreased at high light intensities and oxygen completely represses their formation even in the presence of light. On the other hand, incubation in the dark under low aeration did not permit synthesis of RuDP carboxylase though pigment is formed. It seems therefore that active carboxylase is made only in an environment which promotes both the formation and the normal functioning of bacteriochlorophyll.

The hypothesis of Cohen-Bazire, Sistrom & Stanier (1957) that pigment synthesis is regulated by the state of oxidation of a carrier in the electron transport chain might also apply to the control of RuDP carboxylase formation; different components in the chain might act in each case. This hypothesis could account both for the effect of light intensity and of oxygen. There is now ample evidence, derived from spectroscopic observations of whole organisms and extracts of photosynthetic bacteria, that both illumination and aeration affect the amounts of oxidized and reduced forms of the cytochromes (Frenkel, 1959; Smith & Ramírez, 1959; Smith, Baltscheffsky & Olson, 1960). The amount of DPNH in photosynthetic bacteria is also influenced by illumination (Olson, Duysens & Kronenberg, 1959; Olson & Amesz, 1960). A step towards demonstrating the validity of the hypothesis of Cohen-Bazire *et al.* (1957) would be to correlate one or other of these changes with synthesis of enzymes and pigments. The hypothesis may also be applicable to the adaptive synthesis of RuDP carboxylase in non-photosynthetic bacteria. In the examples studied synthesis of the enzyme is associated with the substrate used as energy source, growth on hydrogen invoking its formation in *Hydrogenomonas* spp. and *Micrococcus denitrificans* (Vishniac & Santer, 1957; Kornberg *et al.* 1960) and on formate in *Pseudomonas oxalaticus* (Quayle & Keech, 1960). The steady state concentrations of reduced and oxidized forms of components of the electron transport chain may differ according to the substrate being oxidized and such differences have, in fact, been shown spectroscopically in *Hydrogenomonas ruhlandii* oxidizing hydrogen or lactate (Packer, 1958).

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The Formation of Isocitratase by the Athiorhodaceae

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SUMMARY

Isocitratase, which cleaves isocitrate to glyoxylate and succinate and is a key enzyme in the glyoxylate cycle, is formed by *Rhodopseudomonas palustris* and *R. capsulatus* when grown on acetate or butyrate either anaerobically in light or aerobically in the dark. Only traces of the enzyme are present in organisms grown on succinate or malate. In contrast, isocitratase is detectable in traces only in *R. spheroides* and *Rhodospirillum rubrum* grown on acetate, butyrate or other substrates. This suggests that the glyoxylate cycle cannot account for net synthesis of cell constituents from acetate or acetate precursors in these latter two organisms.

INTRODUCTION

Many photosynthetic bacteria of the Athiorhodaceae family can grow on bicarbonate+acetate, butyrate or other fatty acids as carbon source (van Niel, 1944), and the catabolism of these organic substrates has been shown by various techniques to proceed via the tricarboxylic acid cycle (Glover, Kamen & van Genderen, 1952; Eisenberg, 1953; Elsden & Ormerod, 1956). The synthesis of cellular materials during growth demands the occurrence of reactions which effect the net formation of intermediates of the tricarboxylic acid cycle from the growth substrates. In many micro-organisms (reviewed by Kornberg, 1959) this is accomplished via the glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957; 1958), a key enzyme of which, isocitratase (Smith & Gunsalus, 1954, 1955, 1957; Olson, 1954, 1959; Saz & Hillary, 1956), is formed only when such micro-organisms grow on acetate or precursors of acetate (Smith & Gunsalus, 1955; Callely, Dagley & Hodgson, 1958; Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959). The main purpose of this paper is to show that, of the Athiorhodaceae examined, *Rhodopseudomonas palustris* and *R. capsulatus* form isocitratase adaptively when grown on acetate or on precursors of acetate, both anaerobically in light and aerobically in the dark. Since the organisms also contain malate synthetase (Wong & Ajl, 1956) the glyoxylate cycle can operate in these organisms under these conditions. However, although *R. spheroides* and *Rhodospirillum rubrum* also contain malate synthetase, they are virtually devoid of isocitratase under all conditions tested, and must therefore differ in the pathways of biosynthesis from acetate from all other micro-organisms examined.

METHODS

Organisms. The following strains of Athiorhodaceae were used: *Rhodopseudomonas spheroides* NCIB no. 8253, *Rhodospirillum rubrum* NCIB no. 8255, *Rhodopseudomonas capsulatus* and *R. palustris* no. 2.3.11 and no. 2.1.7, respectively, from the collection of Professor C. B. van Niel. Stock cultures of all organisms were maintained as described previously (Lascelles, 1956).

Media. The basal medium used contained (mg./l.): KH_2PO_4 and K_2HPO_4, 500 each; $(NH_4)_2HPO_4$, 800; $MgSO_4.7H_2O$, 200; $CaCl_2$, 40; $MnSO_4.4H_2O$, 4; ferric citrate, 3; nicotinic acid and thiamine hydrochloride, 1 each; biotin, 0·01. For growth of *Rhodopseudomonas palustris*, *p*-aminobenzoic acid (0·15 μg./l.) was also added. The basal medium was supplemented as required with DL-malate (20 mM), succinate (10 mM), acetate (20 mM) or butyrate (10 mM), all added as the sodium salts. Sodium bicarbonate (6 mM) was included in all media containing acetate and butyrate as carbon source; the stock solution (0·6 M) was sterilized separately by autoclaving and added aseptically after equilibrating with CO_2. Media were sterilized by autoclaving at 121° for 10 min.

Growth tests. These were carried out in 10 ml. volumes of media in tubes of 15 mm. diameter. Inoculation was with 0·1 ml. of a 1/100 dilution (about 4×10^6 organisms) of a 24 hr. culture grown anaerobically in light on a medium containing malate, glutamate and yeast extract (Lascelles, 1956). Anaerobiosis was achieved by addition of a few crystals of pyrogallol and a drop of saturated K_2CO_3 to the cotton plug which was forced below the rim of the tubes; they were then sealed with rubber bungs. Tubes were incubated at 32° to 34° at 20 cm. distance from a bank of 60 W. tungsten lamps.

Growth and preparation of organisms for cell-free extracts. Cultures were grown anaerobically in light in 8 oz. medical flat bottles containing 200 ml. of medium and stoppered with a rubber bung. The inoculum and details of incubation were described by Lascelles (1956). Aerobic cultures of *Rhodopseudomonas spheroides* were grown in 500 ml. medium in 2 l. Erlenmeyer flasks; the other organisms were grown in 900 ml. medium in flasks of the same size. Inoculation was with 2–5 ml. of 24 hr. culture developed anaerobically in the light in a medium containing malate, glutamate and yeast extract (Lascelles, 1956). Incubation was at 32° in a gyrorotary shaker (New Brunswick Instrument Co., New Jersey, U.S.A.) at 180 oscillations/min.

Cultures were harvested during or towards the end of the logarithmic phase of growth, the incubation time varying between 18 and 24 hr. for photosynthetic cultures and from 24 to 48 hr. for aerobic ones. Organisms were harvested by centrifugation, washed with one-half the original culture volume of 0·02 M-potassium phosphate buffer (pH 7·0), and finally suspended in the same buffer (40 mM) to give a concentration of organisms equivalent to 1 to 10 mg. dry wt./ml.

Preparation of extracts. These were prepared by ultrasonic vibration as described before (Lascelles, 1959). The disrupted preparations were spun at 0° for 10 min. at 25,000 *g* and the supernatant fluid used in the enzyme assays.

Assay of enzymes. Isocitratase and malate synthetase were measured spectrophotometrically by the procedure of Dixon & Kornberg (1959). The activity of isocitratase is expressed as the specific activity (μmole glyoxylate formed in the standard assay/hr./mg. protein).

Estimation of protein and bacteriochlorophyll. These were determined as described previously (Lascelles, 1959).

Measurement of growth. This was determined by turbidity measurements with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex, England) with filter no. 608. Results are expressed as mg. dry wt. organism/ml. culture and were calculated from calibration curves prepared with suspensions of each organism grown anaerobically in light or aerobically in the dark.

RESULTS

Effect of carbon source on growth and bacteriochlorophyll formation

All the organisms grew anaerobically in light on acetate, butyrate, succinate or malate (Table 1). Bicarbonate was necessary for consistent growth from small inocula on acetate or butyrate but did not affect growth on the dicarboxylic acids. Except for *Rhodopseudomonas spheroides* the carbon source did not greatly influence the level of bacteriochlorophyll in the organisms. In *R. spheroides* considerable variation was found, the pigment concentration in organisms grown on acetate being only one-tenth of that in those grown on malate (Table 1). The same substrates also supported aerobic growth (in the dark) of all the organisms.

Table 1. *Growth and bacteriochlorophyll formation on various carbon sources by* Rhodospirillum rubrum, Rhodopseudomonas spheroides, R. capsulatus *and* R. palustris

Cultures were incubated anaerobically in light for 72 hr. in 10 ml. volumes as described in the Methods. The concentrations of organic acids were as follows: succinate and butyrate, 10 mM, acetate; DL-malate, 20 mM. In addition, $NaHCO_3$ (to 6 mM) was added to all tubes after autoclaving. *p*-Aminobenzoic acid (to 1 μM) was included in the basal medium for *Rhodopseudomonas palustris*.

Organism	Carbon source	Growth (mg. dry wt./ml.)	Bacteriochlorophyll (μm-mole/mg. dry wt. organism)
Rhodospirillum rubrum	Succinate	0·68	12·4
	Malate	1·21	13·1
	Acetate	0·67	14·4
	Butyrate	0·12	15·8
Rhodopseudomonas spheroides	Succinate	0·72	5·4
	Malate	1·23	12·0
	Acetate	0·66	1·2
	Butyrate	0·48	4·4
R. capsulatus	Succinate	0·78	22·4
	Malate	0·63	14·8
	Acetate	0·63	16·0
	Butyrate	0·98	10·8
R. palustris	Succinate	0·78	9·1
	Malate	0·98	11·0
	Acetate	0·86	12·8
	Butyrate	1·07	14·0

Isocitratase activity in extracts

Extracts prepared from *Rhodopseudomonas spheroides* grown on acetate or butyrate either anaerobically in light or aerobically in the dark showed only traces of isocitratase. This activity was quite insufficient to contribute significantly to the ability of the organism to utilize these substrates for growth and was no higher than that found in malate-grown organisms. Extracts from organisms which had been successively transferred three times through acetate or butyrate medium were also inactive. Extracts of *Rhodospirillum rubrum* also had negligible isocitratase activity (Table 2).

Table 2. *Isocitratase activity in extracts of Athiorhodaceae*

Extracts were prepared from organisms harvested during the logarithmic phase of growth on the various substrates and were assayed as described in Methods.

Organism	Growth substrate	Isocitratase in culture grown: Anaerobic + light	Aerobic
		(μmole glyoxylate/hr./mg. protein)	
Rhodopseudomonas capsulatus	Acetate	1·90	1·37
	Butyrate	1·58	2·11
	Succinate	0·03	0·04
R. palustris	Acetate	5·4	5·6
	Butyrate	3·4	2·4
	Malate	0·07	—
R. spheroides	Acetate	0·01	0·01
	Butyrate	0·06	0·04
	Malate	0·04	0·03
Rhodospirillum rubrum	Acetate	0·07	0·08
	Malate	0·05	0·04

Isocitratase activity was found in *Rhodopseudomonas capsulatus* and *R. palustris* when grown on acetate and butyrate (Table 2). The specific activity of the isocitratase did not differ significantly in organisms grown anaerobically in light or aerobically in the dark (Table 2). Under both conditions and with both organisms it varied with the physiological age of the organisms at harvesting, reaching a maximum during the logarithmic phase of growth and then declining (Fig. 1).

As found in other organisms (see review, Kornberg, 1959) isocitratase in *Rhodopseudomonas capsulatus* and *R. palustris* was adaptive, being formed only in organisms grown on acetate or butyrate (Table 2). In *R. palustris* addition of succinate to a culture growing on butyrate delayed development of isocitratase; synthesis of this enzyme was resumed after a period during which the added succinate was presumably utilized (Fig. 2). Suppression of isocitratase by succinate has been observed with other organisms (Kornberg *et al.* 1958; Kornberg, Collins & Bigley, 1960).

Malate synthetase. Activity of this enzyme was found in all extracts of all organisms tested under all conditions of growth. Malate synthetase appears to be constitutive in Athiorhodaceae as it is in *Pseudomonas* spp. (Kornberg & Lund, 1959).

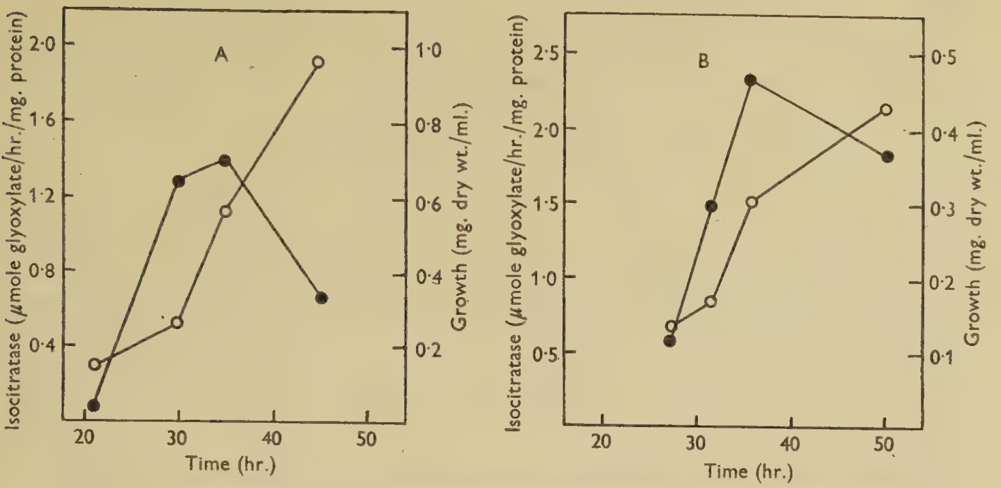

Fig. 1. Isocitratase activity of *Rhodospeudomonas palustris* at various stages of growth. Cultures were grown: anaerobically in light (A); aerobically in the dark (B) on butyrate medium. Samples were removed at intervals for determination of isocitratase activity (—●—) and dry wt. (—○—).

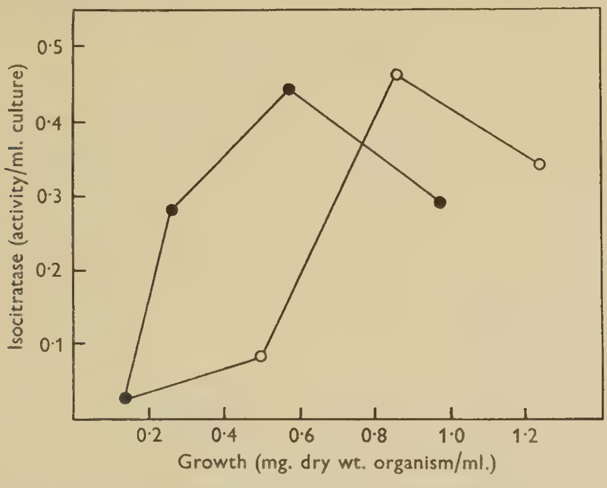

Fig. 2. Effect of succinate on the development of isocitratase in cultures of *Rhodopseudomonas palustris*. A culture growing anaerobically in light in butyrate medium was divided at the beginning of the exponential phase. Succinate (to 5 mM) was added to one part and isocitratase activity determined at intervals thereafter (—○—). No addition was made to the other portion (—●—). Total isocitratase activity is expressed as the product of the specific activity and the suspension density and is plotted against the increase in culture density.

DISCUSSION

The tricarboxylic acid cycle, which has been shown to operate in members of the Athiorhodaceae, is sufficient as a means of providing both energy and the carbon skeletons of cell constituents when succinate or malate is the sole carbon source; but this cycle alone cannot account for growth on acetate or butyrate. In many types of micro-organisms the required synthesis of intermediates of the tricarboxylic acid cycle is effected via the glyoxylate cycle (see Kornberg, 1959). The ability to form isocitratase is a prerequisite for the operation of the glyoxylate cycle. It is therefore of some interest that isocitratase was not found in significant amounts in either *Rhodospirillum rubrum* or in *Rhodopseudomonas spheroides* when grown on acetate or butyrate, though it was present in *R. capsulatus* and *R. palustris* grown under the same conditions. The isocitratase-negative organisms did not appear to be at any obvious disadvantage to the isocitratase-positive organisms with respect to growth rate on these substrates, though the amount of bacteriochlorophyll formed in the light by *R. spheroides* on acetate or butyrate was markedly less than that formed by malate-grown cultures. Since *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* cannot use the glyoxylate cycle they must have other means for obtaining net synthesis of tricarboxylic acid cycle intermediates from acetate. The fact that added bicarbonate is necessary for growth of photosynthetic bacteria on fatty acids (van Niel, 1944) may be significant although, since only negligibly small amounts of isotope from added $NaH^{14}CO_3$ are incorporated by *R. spheroides* aerobically metabolizing acetate, CO_2 is unlikely to act directly as a main precursor of cellular materials under these conditions (Kornberg, to be published). Moreover, the operation of a reductive pentose cycle (Bassham & Calvin, 1957), which could provide a net synthesis of tricarboxylic acid cycle intermediates, cannot account for aerobic growth of the isocitratase-negative organisms on acetate, since ribulose 1:5-diphosphate carboxylase is formed only by cultures growing anaerobically in light (Lascelles, 1960).

The role of the glyoxylate cycle in the isocitratase-positive organisms (*Rhodopseudomonas palustris*, *R. capsulatus*) cannot be quantitatively assessed from the present information. The enzyme was found only in organisms grown on acetate or butyrate and its formation was suppressed by succinate. Such behaviour is typical of organisms known to use the glyoxylate cycle. On the other hand isocitratase was formed by cultures growing anaerobically in light, where the cycle would not be required for net synthesis of tricarboxylic acid cycle intermediates, since these could be formed via the reductive pentose cycle. It is thus possible that, under these conditions, both biosynthetic routes operate simultaneously.

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The Effect of Inhibitors on the Formation of Flagella by *Salmonella typhimurium*

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SUMMARY

The synthesis of flagella by *Salmonella typhimurium* was used in a study of factors affecting the formation of a single protein. Regeneration of flagella by mechanically deflagellated organisms was completely inhibited by 2:4-dinitrophenol (0·001 M), but not by sodium azide (0·004 M), or by sodium arsenate (0·002 M). Ultraviolet (u.v.) irradiation or treatment with nitrogen mustard in a dose sufficient to result in a 99·99 % decrease in viable count, completely inhibited regeneration of flagella. None of the amino acid, purine or pyrimidine analogues tested, with the exception of *p*-fluorophenylalanine, prevented the regeneration of functional flagella, although many of them inhibited bacterial growth. Flagella synthesized in the presence of *p*-fluorophenylalanine were non-functional and the flagella wavelength was half that of the control. Growth of *S. typhimurium* at 44° resulted in a progressive decrease in the number of flagella/bacterium. On continued incubation at 44° the culture became non-motile. Subsequent incubation at 37° resulted in the recovery of motility after a lag about equal to the mean generation time of the bacteria. Addition of 2-thiouracil or 8-azaguanine to a non-motile culture immediately after transferring from 44° to 37° prevented the recovery of motility. When the addition of 8-azaguanine was delayed for 1 hr. after transferring from 44° to 37°, there was no inhibition of recovery of motility. The growth of *S. typhimurium* at 37° was not completely inhibited by 2-thiouracil (0·001 M) or 8-azaguanine (0·001 M), but there was a fall in no. flagella/bacterium after the addition of the analogue. Since regeneration of flagella occurred at 44°, these data were interpreted as indicating that the systems responsible for the formation of flagella were absent from cultures grown at 44°, but were synthesized during the lag before the appearance of flagella on transferring the culture to 37°.

INTRODUCTION

Mechanical removal of flagella from *Salmonella typhimurium* results in the bacteria becoming non-motile, but has no effect on their viability (Stocker, 1957; Stocker & Campbell, 1959); on incubation under suitable conditions these bacteria are able to regenerate flagella and so regain their motility. Since flagella from *S. typhimurium* consist of a single well-characterized protein (Kauffmann, 1954; Ambler & Rees, 1959), regenerating flagella provide a suitable system for studying protein synthesis (Kerridge, 1959*a*). Quadling & Stocker (1956) found that it was possible to transfer a logarithmically growing culture of *S. typhimurium* from an environment in which flagellar formation occurred normally, to one in which formation of flagella ceased although growth continued. It is possible, by continued incubation under conditions unfavourable for the synthesis of flagella, to obtain a culture of non-motile organisms. These bacteria do not lose the potentiality for the production of flagella and on transfer to favourable conditions regain their motility after a short lag.

Inhibitors play an important role in any investigation of the mechanisms of protein synthesis by micro-organisms. Certain amino acid analogues are incorporated into bacterial protein, often with the production of biologically inactive protein (Munier & Cohen, 1956, 1959; Baker, Johnson & Fox, 1958). Since Matthews (1953) showed that 8-azaguanine is incorporated into the ribonucleic acid (RNA) of tobacco mosaic virus, numerous workers have studied the effect of purine and pyrimidine analogues on nucleic acid and protein synthesis and their interrelationship. In *Staphylococcus aureus*, the formation of the inducible enzymes β-galactosidase and catalase was found to be more sensitive to inhibition by 8-azaguanine than the constitutive glucozymase system (Creaser, 1956). Richmond (1959), who studied enzyme formation in *Bacillus subtilis* R, found that both inducible β-galactosidase and constitutive lytic enzyme formation were equally sensitive to inhibition by 8-azaguanine.

Cultures of *Salmonella typhimurium* can be made non-flagellate by either the mechanical removal of their flagella or by growth at 44°, at which temperature flagella are not produced. The first section of this paper describes an investigation of the effects of inhibitors on the regeneration of flagella by mechanically deflagellated bacteria; the second section describes their effects on the recovery of motility at 37° by *S. typhimurium* grown at 44°. Preliminary reports of this work have been given (Kerridge, 1959).

METHODS

Organisms and growth media. The strains of *Salmonella typhimurium* used are detailed in Table 1. The cultures were maintained on Dorset's egg slopes at room temperature.

Table 1. *Characteristics of strains of* Salmonella typhimurium *used*

Strain	Parent strain	Characteristic	Lab. ref. no.	Source
LT2	—	Wild-type	LT2	Zinder & Lederberg (1952)
SW1061	LT2	Monophasic H (1.2.3.)	SW1061	Dr T. Iino
SW577	—	90 % 'curly' flagella	SW577	Dr E. Leifson
462	553	Tyrosine requiring	SL185	Dr H. Plough
try A–8	LT2	Tryptophan requiring	SL282	Demerec *et al.* (1956)
phe-5	LT2	Phenylalanine requiring	SL448	Demerec *et al.* (1956)

Unless otherwise stated, *Salmonella typhimurium* strain SW1061 was used for the experimental work.

For the preparation of washed suspensions, organisms were grown in a nutrient broth made from a pancreatic digest of casein, dispensed in 150 ml. quantities in Roux bottles. The inoculum was 3 ml. of an overnight culture of the organism in nutrient broth. The bacteria were harvested by centrifugation after incubation for 4 hr. at 37°. The organisms were washed twice with saline (0·85 %, w/v) and resuspended in saline at a concentration equivalent to 10 mg. dry wt./ml. The equiv. dry weights of the organisms in suspension were estimated by using a Hilger 'Spekker' absorptiometer previously calibrated for the organisms. The flagella were detached mechanically from the bacteria as previously described (Kerridge, 1959*a*).

Non-motile cultures of *Salmonella typhimurium* were obtained by growing the bacteria at 44° in a medium consisting of (%, w/v): K_2HPO_4, 0·7; KH_2PO_4, 0·2; Na_3 (citrate) $5H_2O$, 0·05; $MgSO_47H_2O$, 0·01; $(NH_4)_2SO_4$, 0·01; Difco vitamin-free Casamino acids, 0·2; glucose, 0·2.

Regeneration of bacterial flagella. Regeneration of flagella was carried out in 250 ml. conical flasks containing (%, w/v): K_2HPO_4, 0·7; KH_2PO_4, 0·2; Na_3 (citrate) $5H_2O$, 0·05; $MgSO_47H_2O$, 0·01; glucose, 0·2; the following amino acids, each at a concentration of 0·1 mg. of the L isomer/ml.: glycine, serine, glutamic acid, aspartic acid, valine, leucine, isoleucine, arginine, proline, alanine, histidine, lysine, tyrosine, cysteine, threonine, methionine, ornithine, tryptophan and phenylalanine. When inhibitors other than amino acid analogues were being investigated the amino acid mixture was replaced by Difco vitamin-free Casamino acids (0·2 %, w/v). Organisms were added to a final concentration equivalent to 0·2 mg. dry wt./ml.; 100 ml. medium was incubated at 37°.

Viable counts were performed by the method described by Miles & Misra (1938) after serial dilution in saline.

Regeneration of flagella was determined both by motility estimations and flagellar staining (Kerridge, 1959*a*).

Nucleic acid and protein synthesis. The organisms in 10 ml. samples were harvested by centrifugation, resuspended in 2·0 ml. 5 % (w/v) trichloroacetic acid (TCA) and held at 4° for 2 hr. The precipitate was harvested by centrifugation and washed twice with cold 0·2 N-perchloric acid. Nucleic acid was extracted from the precipitate with 0·5 N-perchloric acid; two 20 min. extractions at 70° were sufficient to remove all the nucleic acid. The supernatant fluids from the extractions were combined and made up to 5·0 ml. with 0·5 N-perchloric acid. Total nucleic acid was determined after suitable dilution, by measuring the extinction at 260 mμ with a Beckman model DU spectrophotometer, and the deoxyribonucleic acid (DNA) by the diphenylamine method of Burton (1956). The residue after the extraction of nucleic acid was taken up in 5·0 ml. N-NaOH and the protein content estimated colorimetrically with Folin's reagent, by the method of Lowry, Rosebrough, Farr & Randall (1951).

Incorporation of ^{14}C-*labelled amino acids.* In certain experiments the incorporation of a ^{14}C-labelled amino acid was used as a measure of nucleic acid and protein synthesis. The ^{14}C-labelled amino acid was added to the regeneration medium at a final concentration of 0·05 μc./ml. Organisms in 1 ml. samples were harvested by centrifugation, resuspended in 1 ml. 5 % (w/v) TCA and held at 4° for 2 hr. The precipitate was washed twice with cold 5 % (w/v) TCA. The nucleic acid was extracted from the precipitate with TCA, one 30 min. extraction at 100° with 2·0 ml. 5 % (w/v) TCA being sufficient to remove all the nucleic acid. The precipitate was removed by centrifugation and the supernatant fluid extracted three times with an equal volume of ether to remove the TCA. The resulting aqueous solution was evaporated to dryness, the residue taken up in N-NH_4OH and transferred to a metal planchette, containing a piece of lens tissue and 0·03 ml. of 0·1 % (w/v) aqueous cetyl trimethylammonium bromide to facilitate the spreading of the sample. The residue after the extraction of the nucleic acid was taken as the protein fraction; this was suspended in N-NH_4OH and transferred to a metal planchette. After drying, the radioactivity of the planchette contents was determined by using a Panax type D657 counter in association with a thin mica end-window Geiger-Muller tube. Counting was continued for 20 min., or until 1000 counts had been recorded.

Induction and estimation of nitratase activity in washed suspensions of Salmonella typhimurium. Induction was carried out anaerobically in Thunberg tubes at 37° in a system consisting of 0·05 M-phosphate buffer (pH 7·0); 0·01 M-KNO_3; amino acid mixture as for the regeneration of flagella; 2·0 % (w/v) glucose; organisms to a final concentration equiv. 0·4 mg. dry wt./ml. After incubation for 2 hr. the organisms were harvested by centrifugation, washed once with distilled water, suspended in distilled water and the concentration determined turbidimetrically. The nitratase activity was determined by the method of Pollock (1946).

Preparation of bacterial flagella. The bacteria were harvested by centrifugation, resuspended in distilled water at a concentration equiv. 100 mg. dry wt./ml. and the flagella detached mechanically (Kerridge, 1959*a*). Bacteria were removed by centrifugation for 15 min. at 10,000 *g*. The supernatant fluid was dialysed overnight at 4° against distilled water, and the flagella harvested by centrifugation for 40 min. at 105,000 *g* in a Spinco Model L centrifuge. The pellet was taken up in distilled water and after removal of contaminating material by centrifuging at 30,000 *g* for 1 min., the flagella were harvested by centrifugation at 105,000 *g* for 40 min.

Amino acid analysis. The flagellin was hydrolysed in 6N-HCl in sealed tubes for 16 hr. at 105°. After removal of HCl by distillation under reduced pressure the hydrolysate was chromatographed, using the two-solvent system of Boissonas (1950). The amino acids were detected by spraying with 0·01 % (w/v) ninhydrin in acetone and the colour developed by heating at 105° for 5 min.

RESULTS

Effect of various inhibitors on protein, DNA and total nucleic acid synthesis and on the regeneration of flagella by mechanically deflagellated bacteria

Miscellaneous inhibitors. In a study of factors affecting the motility of *Proteus vulgaris*, de Robertis & Peluffo (1951) found that motility was inhibited by *p*-chloromercuribenzoate (0·01 M), but not by 0·01 M-potassium cyanide, sodium azide, sodium fluoride or sodium malonate. In the present work the motility of *Salmonella typhimurium* in a nutrient broth was unaffected by 2:4-dinitrophenol (0·002 M), but there was a slight decrease in the proportion of motile bacteria when incubated with sodium arsenate (0·001 M). Although these compounds had little effect on motility, they inhibited regeneration of flagella by mechanically deflagellated *S. typhimurium*. Addition of 2:4-dinitrophenol (0·001 M) to the regeneration medium resulted in the complete inhibition of synthesis of nucleic acid, protein and flagella. Lower concentrations of 2:4-dinitrophenol affected synthesis of flagella and total protein synthesis to about the same extent. Regeneration of flagella was less sensitive to inhibition by sodium arsenate than general protein synthesis: 0·001 M-sodium arsenate completely inhibited net nucleic acid and protein synthesis, but approximately 50 % of the bacteria regenerated flagella. Even in the presence of 0·002 M-sodium arsenate some regeneration of flagella occurred. Sodium azide (0·004 M) did not completely inhibit the synthesis of nucleic acid, protein or flagella by *S. typhimurium* (Table 2).

Nitrogen mustard (Harold & Ziporin, 1958) and ultraviolet radiation (Kelner, 1953) preferentially inhibit DNA synthesis in micro-organisms; at high dosage, inhibition of RNA and protein synthesis also occurs. In the present work treatment of washed suspensions of *Salmonella typhimurium* in saline (0·85 %, w/v) with nitrogen mustard (di-(2-chloroethyl) methylamine HCl) had a marked inhibitory effect on nucleic acid and protein synthesis when the organisms were subsequently incubated in the regeneration medium. Incubation of a washed suspension of *S. typhimurium* (at equiv. 10 mg. dry wt./ml.) with 500 μg. nitrogen mustard/ml. for 10 min. at room temperature resulted in a 99·9 % decrease in the viable count (Table 3). On subsequent incubation in the regeneration medium, DNA synthesis was completely inhibited and RNA and protein synthesis were 67 and 15 % inhibited, respectively. Regeneration of flagella was almost completely inhibited, for only 8 % of the bacteria regenerated flagella during the 2 hr. incubation period. Treatment with lower concentrations of nitrogen mustard resulted in less pronounced effects on nucleic acid and protein synthesis. At 100 μg. nitrogen mustard/ml. there was no diminution in protein synthesis during the incubation, but synthesis of flagella was appreciably inhibited. Only 40 % of the bacteria regenerated flagella as compared with 83 % in the control without nitrogen mustard (Table 3).

Table 2. *The effect of inhibitors on the regeneration of flagella, and on protein and nucleic acid synthesis by* Salmonella typhimurium *strain SW* 1061

		Bacterial motility after 2 hr. aerobic incubation at 37° in the complete regeneration medium			Inhibition (%) of synthesis after aerobic incubation for 2 hr. in the complete regeneration medium		
Inhibitor	Concn. (M)	Motile bacteria (%)	Flagellate bacteria (%)	Mean no. flagella/ flagellate bacterium	Total nucleic acid	DNA	Protein
2:4-dinitrophenol	0	90	86	3·6	.	.	.
	0·0005	50	35	2·1	80	70	84
	0·001	1	.	.	100	100	94
	0·002	1	.	.	100	100	100
Na arsenate	0	70	70	3·5	.	.	.
	0·0005	50	49	1·8	90	70	95
	0·001	50	45	1·7	97	100	100
	0·002	50	44	1·5	100	100	100
Na azide	0	80	.	.	.	.	.
	0·002	60	.	.	41	43	71
	0·004	50	.	.	56	68	60

Ultraviolet irradiation with a dose sufficient to give a 99·9 % decrease in the number of viable bacteria completely inhibited the net synthesis of nucleic acid and protein; regeneration of flagella was not completely inhibited and after incubation for 2 hr., 50 % of the bacteria had regained motility (Table 3).

Antibiotics. Since Fitzgerald, Bernheim & Fitzgerald (1948) reported that the synthesis of the enzyme system involved in the adaptive utilization of benzoic acid by *Mycobacterium lacticola* was inhibited by streptomycin, numerous other antibiotics have been shown to affect nucleic acid and protein metabolism in microorganisms. Chloramphenicol, aureomycin and terramycin, at the minimum growth inhibitory concentration, completely inhibit the formation of protein by washed suspensions of *Staphylococcus aureus* (Gale & Paine, 1951; Gale & Folkes, 1953). Chloramphenicol has a similar effect on protein and nucleic acid synthesis in *Escherichia coli* (Hahn & Wisseman, 1951). Penicillin inhibits the formation of bacterial cell wall (Park & Strominger, 1957), and it has been claimed recently that streptomycin damages the cell membrane in *E. coli* (Anand & Davis, 1960).

Table 3. *The effect of nitrogen mustard and u.v. irradiation on the regeneration of flagella and on nucleic acid and protein synthesis by* Salmonella typhimurium *strain SW* 1061

		Bacterial motility after 2 hr. aerobic incubation at 37° in the complete regeneration medium				Inhibition (%) of synthesis after aerobic incubation for 2 hr. in the complete regeneration medium		
Inhibitor	Concn. (µg./ml.)	Residual viable cells* (%)	Motile bacteria (%)	Flagellate bacteria (%)	Mean no. flagella/ flagellate bacterium	Total nucleic acid	DNA	Protein
Nitrogen mustard†	0	7	90	83	3·9	.	.	.
	100	7	30	40	2·7	22	17	0
	250	2	10	38	2·8	44	67	0
	500	0·1	5	8	2·8	67	100	15
	1000	0·01	1	7	2·6	88	100	16
	Irradiation time (min.)							
u.v. radiation‡	0	.	90	86	2·9	.	.	.
	2	1·0	60	55	2·7	80	80	52
	5	0·1	50	49	2·6	100	100	100
	10	0·01	1	1	.	100	100	100

* The viable count was made at the beginning of the incubation.

† Washed suspensions of *Salmonella typhimurium* were incubated at room temperature with nitrogen mustard before transferring to the regeneration medium.

‡ Irradiation with a Thermal syndicate type T/M5/369 mercury vapour lamp having 95% emission at 2536 A.

If the system involved in the synthesis and functioning of the bacterial flagellum is closely associated with the bacterial membrane or the cell wall, then it is possible that antibiotics which interfere with the formation of these structures might have a secondary effect on flagellar synthesis. Incubation of *Salmonella typhimurium* strain SW 1061 in the presence of growth-inhibitory concentrations of penicillin resulted in bacterial lysis. When the flagella were removed mechanically before the addition of penicillin, regeneration of flagella occurred before cell lysis. The addition of sucrose (20%, w/v) to the regeneration medium stabilized the spheroplasts produced as a result of incubation in the presence of penicillin, and these were shown to possess flagella, both by staining and serological reactions. It was shown previously (Kerridge, 1959*a*) that *S. typhimurium* strain SL 282 (tryptophan-requiring) regenerated flagella in the absence of net protein synthesis when incubated in the absence of tryptophan. In the presence of growth-inhibitory concentrations of penicillin, flagellar regeneration occurred in *S. typhimurium* strain SL282 in the absence of tryptophan without the production of spheroplasts.

Flagella could be detached from penicillin-induced spheroplasts of *Salmonella typhimurium*, but although the spheroplasts were capable of incorporating ^{14}C-labelled aspartic acid into the material insoluble in cold 5% (w/v) TCA when incubated in the regeneration medium supplemented with 20% (w/v) sucrose, synthesis of flagella could not be demonstrated under these conditions.

The addition of a growth-inhibitory concentration of streptomycin to a culture of *Salmonella typhimurium* did not result in the immediate inhibition of motility. At the minimum growth-inhibitory concentration (10 μg./ml.) streptomycin had little effect on flagellar regeneration, and even at 100 μg. streptomycin/ml. the majority of the bacteria regenerated flagella. Both penicillin and streptomycin at the minimum growth-inhibitory concentration had some inhibitory effect on nucleic acid and protein synthesis by *S. typhimurium* (Table 4).

Table 4. *The effects of antibiotics on the regeneration of flagella, and on nucleic acid and protein synthesis by* Salmonella typhimurium *strain SW* 1061

		Bacterial motility after aerobic incubation for 2 hr. at 37° in complete regeneration medium			Inhibition (%) of synthesis after aerobic incubation for 2 hr. in the complete regeneration medium		
		Motile bacteria (%)	Flagellate bacteria (%)	Mean no. flagella/ flagellate bacterium	Total nucleic acid	DNA	Protein
Penicillin (units/ml.)	0	100	82	3·2	.	.	.
	10	70	85	5·7	3	20	20
	30	70	85	5·7	63	63	40
Streptomycin (μg./ml.)	0	90	.	.	.	.	.
	30	90	.	.	12	0	0
	100	70	.	.	72	81	61

Amino acid analogues. The relative ease of isolation of bacterial flagella makes flagellin synthesis a suitable system for the study of the incorporation of amino acid analogues into a single functional protein; minor changes in the protein which might occur also can be studied serologically. The effects of various amino acid analogues on growth and synthesis of flagella by *Salmonella typhimurium* were studied. Following addition of the analogues *p*-fluorophenylalanine (*p*-FP), β-2-thienylalanine or ethionine to cultures of *S. typhimurium* growing logarithmically in a minimal medium, there was an immediate decrease in the growth rate (Fig. 1). Microscopic examination of the cultures at the end of the experiment showed that the bacteria were motile after incubation with β-2-thienylalanine or ethionine; long forms were present in the culture incubated with β-2-thienylalanine. Organisms incubated with *p*-FP became non-motile, and staining demonstrated the presence of abnormal flagella (Kerridge, 1959*b*).

In a previous paper (Kerridge, 1959*a*) an investigation of flagellar regeneration by auxotrophic mutants of *Salmonella typhimurium* was reported. The effects of amino acid analogues on flagellar regeneration by certain of these strains was now studied and the results are shown in Table 5. *S. typhimurium* strain SW577 was of interest as the flagella in this organism are aberrant, having a flagellar wavelength (1·0 μ) half that usually found in *S. typhimurium* and the organisms are non-motile. Strain SW 577a is a motile variant isolated from strain SW 577, and the wavelength of the flagella is normal. After mechanical removal of flagella, bacteria of both strains regenerated flagella when incubated in a complete medium. Replacement of the phenylalanine of this medium by *p*-FP (0·005 M) had no apparent effect on flagellar regeneration by strain SW 577 (non-motile), but with strain SW 577a (motile) the flagella synthesized in the presence of *p*-FP were morphologically similar to those of strain SW 577 and were non-functional. *p*-FP and *β*-2-thienylalanine were unable to replace tyrosine for flagellar regeneration by the tyrosine-requiring strain SL 185, but replaced phenylalanine for strain SL 448, abnormal flagella with a wavelength half that of the control being produced in the presence of *p*-FP. Replacement of the phenylalanine of the regeneration medium by *p*-FP (0·004 M) had no effect on the net synthesis of nucleic acid or protein by *S. typhimurium* strain LT 2 during incubation for 90 min., although there was a marked effect on the synthesis of functional flagella. Unlike *p*-FP, *β*-2-thienylalanine inhibited the net synthesis of nucleic acid and protein (Table 6), but synthesis of functional flagella occurred in the presence of *β*-2-thienylalanine. Addition of *p*-FP to motile cultures of *S. typhimurium* did not affect flagellar morphology or function; removal of the analogue from cultures with aberrant flagella and replacement by phenylalanine did not result in a reversion to the normal type of flagella. Chromatographic analysis (Boissonas, 1950) of the acid hydrolysates of the protein fraction and of flagella isolated from organisms incubated in the presence of *p*-FP showed the presence of the analogue in the protein.

Table 5. *The effect of amino acid analogues on growth and on the regeneration of flagella by amino acid-requiring strains of* Salmonella typhimurium

The regeneration of flagella was carried out in the complete regeneration medium, the amino acid required for growth being omitted when the amino acid analogue was added to the medium.

S. typhimurium strain	Characteristic	Analogue (concn.)	Growth	Motile bacteria after aerobic incubation for 2 hr. at 37° (%)	Flagella
SW 577	'Curly' flagella		+	5	Aberrant
		p-FP (0·005 M)	+	5	Aberrant
SW 577a	Normal variant isolated from strain SW 577		+	50	Normal
		p-FP (0·005 M)	+	10	Aberrant
SL 185	Tyrosine-requiring		+	80	Normal
		p-FP (0·005 M)	−	1	Absent
		β-2-thienylalanine (0·002 M)	−	1	Absent
SL 282	Tryptophan-requiring		+	70	Normal
		− tryptophan	−	60	Normal
		5-methyl tryptophan (0·005 M)		50	Normal
SL 448	Phenylalanine-requiring		+	80	Normal
		p-FP (0·005 M)	±	5	Aberrant
		β-2-thienylalanine (0·002 M)	±	50	Normal

Table 6. *The effect of amino acid analogues on the regeneration of flagella, and on nucleic acid and protein synthesis by* Salmonella typhimurium *strain SW*1061

		Bacterial motility after 2 hr. aerobic incubation at 37° in the complete regeneration medium			Inhibition (%) of synthesis after aerobic incubation for 2 hr. in the complete regeneration medium		
Analogue	Concn. (M)	Motile bacteria (%)	Flagellate bacteria (%)	Mean no. flagella/ flagellate bacterium	Total nucleic acid	DNA	Protein
p-fluorophenylalanine	0	90	96	3·0	.	.	.
	0·01	10	80	2·3	0	0	0
β-2-thienylalanine	0	90	.	.	.	.	.
	0·002	90	.	.	75	20	80
	0·001	85	.	.	50	40	40

Purine and pyrimidine analogues. In *Bacillus cereus*, 8-azaguanine inhibits protein synthesis almost completely while being incorporated into the RNA. Chantrenne & Devreux (1958) compared its effect to that of chloramphenicol and suggested that the same mechanisms might be blocked in both cases. A differential effect of 8-azaguanine on inducible and constitutive enzyme synthesis in *Staphylococcus aureus* was found by Creaser (1956). Flagellin behaves as a constitutive protein in *Salmonella typhimurium* (Kerridge, unpublished results) and it was of interest to study

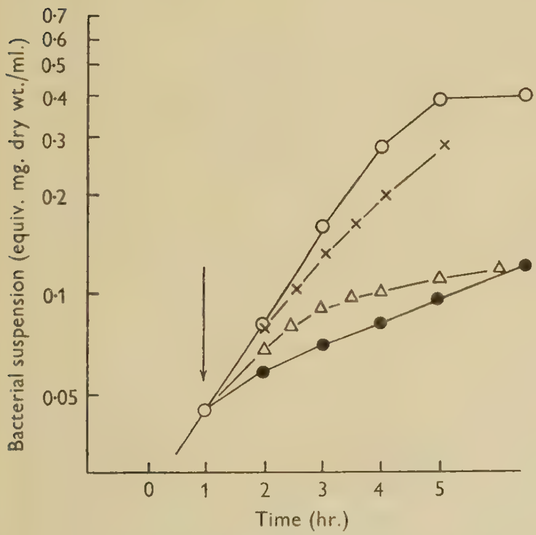

Fig. 1

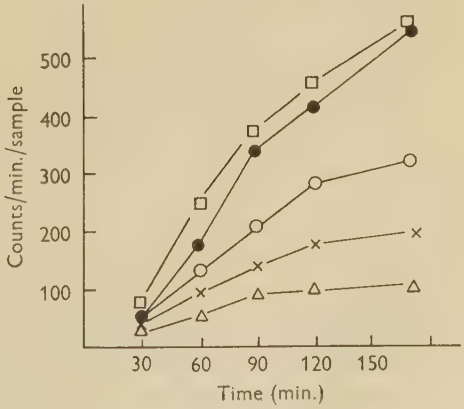

Fig. 2

Fig. 1. The effect of amino acid analogues on the growth of *Salmonella typhimurium*. -○—○- = control; -×—×- = +ethionine (0·005 M); -△—△- = +*p*-FP (0·005 M); -●—●- = +*β*-2-thienylalanine (0·005 M).

Fig. 2. Inhibition of ^{14}C-glycine incorporation into the nucleic acid and protein fractions of *Salmonella typhimurium* by 5-fluorouracil. -●—●- = protein, control; -○—○- = nucleic acid, control; -□—□- = protein + fluorouracil ($7{\cdot}4\times10^{-4}$ M); -△—△- = nucleic acid + fluorouracil ($7{\cdot}4\times10^{-4}$ M); -×—×- = nucleic acid + fluorouracil ($7{\cdot}4\times10^{-5}$ M).

the effects of purine and pyrimidine analogues on the formation of flagella. Flagellar regeneration by *S. typhimurium* was not inhibited by 8-azaguanine, even at $6\cdot5\times10^{-4}$ M. There may have been some effect on the rate of flagellin synthesis, although the data suggest that this was not great. The flagella synthesized in the presence of 8-azaguanine were morphologically normal and were functional. Net protein and nucleic acid synthesis was 50 % inhibited during incubation for 2 hr. with 8-azaguanine ($6\cdot5\times10^{-4}$ M), but DNA synthesis was not as sensitive as RNA synthesis (Table 7). 8-Azaguanine ($6\cdot5\times10^{-5}$ M) inhibited the synthesis of the inducible nitratase system in *S. typhimurium* by 95 %.

Table 7. *The effect of purine and pyrimidine analogues on the regeneration of flagella, and on nucleic acid and protein synthesis by* Salmonella typhimurium *strain SW*1061

		Bacterial motility after 2 hr. aerobic incubation at 37° in the complete regeneration medium			Inhibition (%) of synthesis after incubation for 2 hr. in the complete regeneration medium		
Analogue	**Concn. (M × 10⁻⁴)**	**Motile bacteria (%)**	**Flagellate bacteria (%)**	**Mean no. flagella/ flagellate bacterium**	**Total nucleic acid**	**DNA**	**Protein**
8-Azaguanine	0	90	86	2·5	.	.	.
	0·65	90	94	3·2	32	16	41
	1·9	90	88	2·5	42	10	51
	6·5	90	95	2·8	50	16	51
2-Thiouracil	0	90	84	2·6	.	.	.
	30	.	.	.	46	25	37
	10	90	.	.	85	69	80
	30	90	79	2·4	86	73	88
6-Azauracil	0	85	85	3·7	.	.	.
	0·85	75	84	2·8	68	51	75
	2	70	70	2·6	95	68	87
	4·3	65	65	1·9	92	58	100
	8·5	50	66	2·1	.	.	.
5-Fluorouracil	0	80	80	3·7	.	.	.
	0·23	75	95	6·1	.	.	.
	2·3	80	86	7·6	.	.	.
	7·4	80	95	7·9	.	.	.
FDUR*	0·01	80	66	3·8	.	.	.
	0·1	60	62	4·5	50	81	47
	1·0	60	48	5·2	38	90	36

* Fluorodeoxyuridine.

Hamers (1956) showed that 2-thiouracil was incorporated into the RNA fraction of *Bacillus megaterium*, replacing uracil, and that linear growth occurred in the presence of the analogue. The addition of 2-thiouracil to the regeneration medium had little or no effect on the ability of *Salmonella typhimurium* to regenerate functional flagella, whereas the synthesis of nucleic acid and protein was largely prevented (Table 7). DNA synthesis was inhibited less than either protein or total nucleic acid (RNA) synthesis.

Cohen *et al.* (1958) studied the effects of 5-fluorouracil and its derivatives on the growth of *Escherichia coli* and found that it was incorporated into the RNA of the bacteria. Fluorouracil is also converted to fluorodeoxyuridine (FDUR) which interferes with the functioning of thymidylate synthetase and results in the inhibition of DNA synthesis. FDUR is itself a very good inhibitor of growth of *E. coli*, giving rise to 'unbalanced growth' and resulting in 'thymineless death'. 5-Fluorouracil had a differential effect on the rate of incorporation of ^{14}C-labelled glycine into the nucleic acid and protein fractions of *Salmonella typhimurium* during incubation in the regeneration medium. At a concentration of $7{\cdot}4 \times 10^{-4}$M there was no effect on the rate of glycine incorporation into the protein fraction, but the incorporation of glycine into the nucleic acid fraction was initially slower than in the control and ceased after 90 min. (Fig. 2). Regeneration of flagella was not inhibited and microscopic examination demonstrated that after incubation for 2 hr. in the regeneration medium there was a marked increase in the number of flagella/bacterium (Table 7). In the control culture there were approximately 4 flagella/bacterium, whereas in the culture incubated for 2 hr. in the presence of $7{\cdot}4 \times 10^{-4}$M-5-fluorouracil, there were approximately 8 flagella/bacterium. This effect may have resulted from an inhibition of cell division, without any effect on the formation of flagella. FDUR had a differential effect on the net nucleic acid and protein synthesis, DNA synthesis being more sensitive to inhibition than either RNA or protein synthesis. FDUR had an effect similar to 5-fluorouracil on the regeneration of flagella; incubation in the presence of FDUR (10^{-4}M) resulted in an increase in the mean number of flagella/bacterium. The effect was not as pronounced as in the case of 5-fluorouracil.

6-Azauracil was found by Otsuji & Takagi (1959) to inhibit the synthesis of DNA by *Escherichia coli* K 12 after a lag of *c.* 30 min., while RNA and protein synthesis continue. These authors suggested that the inhibitory effects on growth were not necessarily due to an effect of the incorporation of the analogue into the nucleic acid, but might be due to an inhibition of cell-wall synthesis. A somewhat different result was obtained with *Salmonella typhimurium.* 6-Azauracil (4×10^{-5}M) had no differential effect on net RNA, DNA and protein synthesis during incubation for 2 hr.: at higher concentrations RNA and protein synthesis were more strongly inhibited than DNA synthesis (Table 7). Regeneration of flagella occurred in the presence of $8{\cdot}5 \times 10^{-4}$M 6-azauracil but at this concentration the mean number of flagella/bacterium after incubation for 2 hr. was less than in the control culture. 5-Bromouracil, 5-nitrouracil, 4-methyl-2-thiouracil, isocytosine and 2:6-diaminopurine were also tested; they had no effect on growth or regeneration of flagella by *S. typhimurium.*

Temperature effect; recovery of motility after growth at 44°

Quadling & Stocker (1956) found that it was possible to transfer a logarithmically growing culture of *Salmonella typhimurium* strain LT 2 from 37° to 44° without affecting the growth rate. At 44° there was a progressive decrease in the mean no. flagella/bacterium, and eventually the flagellated bacteria were diluted out. This effect was confirmed in the present work; a typical experimental result is shown in Fig. 3. There was no change in the growth rate of *S. typhimurium* strain SW 1061 on raising the temperature from 37° to 44°. The mean no. flagella/bacterium remained constant for 1 hr. and then there was a progressive decrease in the no. flagella/bacterium. The loss of motility was a phenotypic effect and on transfer to a favourable environment the bacteria recovered their motility.

Logarithmic growth continued after transferring a non-motile culture of *Salmonella typhimurium* from 44° to 37°, but there was no immediate synthesis of flagella. Motile bacteria did not appear until about 45 min. after the transfer and all organisms were not motile until after *c.* 90 min. A comparison of the recovery of motility following growth at 44° with regeneration of flagella after the mechanical removal of the flagella is shown in Fig. 4. The rates of growth and formation of flagella were similar in both cases, but there was a considerable difference in the length of the time lag before the appearance of flagella. It was considered possible that this lag might be due to the time taken by the bacteria to synthesize the system responsible for the formation and functioning of the flagellum. If the lag before the appearance of a flagellum at 37°, following incubation at 44°, represents the time required for the production of the synthesizing system, it should be possible to investigate the factors which affect the formation of a protein-synthesizing system by studying variations in this lag period.

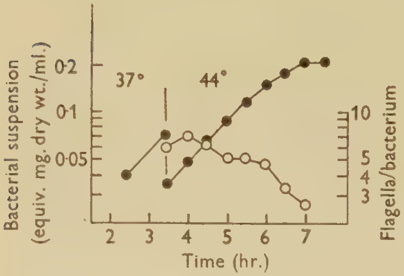

Fig. 3

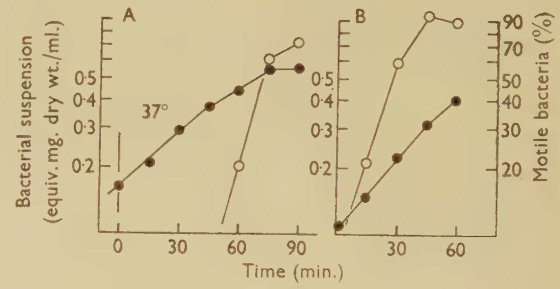

Fig. 4

Fig. 3. The effect of temperature on the growth of *Salmonella typhimurium* and on no. flagella/bacterium. -●—●- = suspension concentration; -○—○- = no. flagella/bacterium.

Fig. 4. A, Growth and recovery of motility at 37° by a non-motile culture of *Salmonella typhimurium* after incubation at 44°; B, growth and flagellar regeneration at 37° after mechanical removal of the flagella. -●—●- = suspension concentration; -○—○- = % motile bacteria.

Inhibition of the recovery of motility after growth at 44°

Effect of purine and pyrimidine analogues. Purine and pyrimidine analogues have no effect on regeneration of flagella in *Salmonella typhimurium*, but since several of these compounds have been found to affect enzyme synthesis in other bacteria, a study was made of their effects on the recovery of motility by *S. typhimurium* after growth at 44°. An overnight culture of *S. typhimurium* in the Casamino acids salts medium was diluted tenfold with similar medium, and incubation continued at 44°. After 4 hr. the culture was diluted with an equal volume of medium and incubated at 37°. Addition of 2-thiouracil (0·001 M) to the culture at the time of transfer to 37° resulted in a progressive inhibition of growth. After incubation for 2·5 hr. at 37° the bacteria remained non-motile and flagella could not be demonstrated by staining. A similar result was obtained using 8-azaguanine. The control culture continued to grow logarithmically after transferring to 37° and after incubation for 2 hr. at least 80 % of the bacteria were motile (Fig. 5). As neither 2-thiouracil nor 8-azaguanine had been shown to have any marked inhibitory effects on regeneration of flagella at 37° after mechanical detachment of the flagella (Table 7), it seemed probable that the inhibition of recovery of motility was not due to an effect on flagellin synthesis itself, but rather to an effect on the formation of the synthesizing system. This hypothesis was studied by investigating the effects of delaying the addition of the analogue.

A non-motile culture of *Salmonella typhimurium* growing logarithmically at 44° was diluted with an equal volume of growth medium and incubated at 37°. Immediately after the dilution and then at 30 min. intervals, 20 ml. samples were taken and 8-azaguanine added (to final concentration 0·001 M) to them. The cultures were incubated at 37° and sampled at intervals for the determination of the quantity of bacteria and their motility (Fig. 6). After the addition of 8-azaguanine the growth rate was decreased and the rate of growth was no longer logarithmic. The recovery of motility was completely inhibited by the analogue when added immediately after transfer to 37°; less than 5 % of the bacteria were motile after 4 hr. at 37° as compared to 90 % in the control culture. Delay in the addition of 8-azaguanine for 1 hr. had little effect on the subsequent formation of flagella, although the growth rate was decreased. At the time of addition of the inhibitor, less than 5 % of the bacteria were flagellate, but 1 hr. later 60 % of the bacteria were motile. Examination of

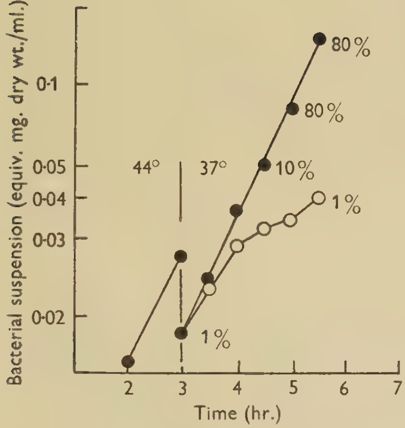

Fig. 5

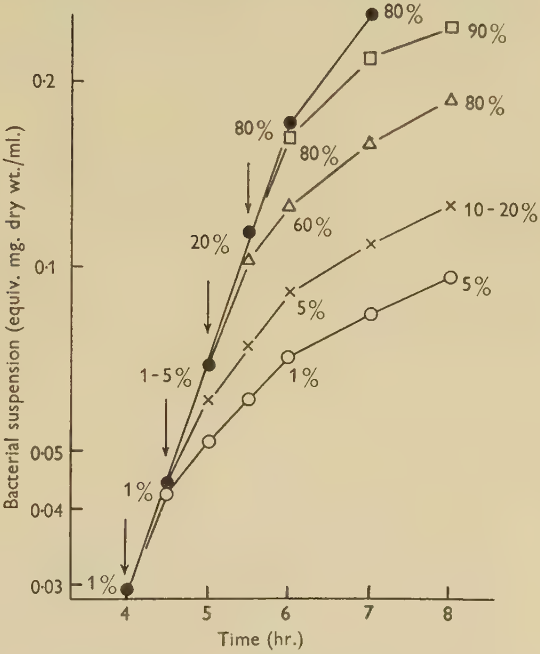

Fig. 6

Fig. 5. The effect of 2-thiouracil on the growth and recovery of motility by a non-motile culture of *Salmonella typhimurium*. -●—●- = control; -○—○- = +2-thiouracil (0·001 M). The numbers denote % motile bacteria.

Fig. 6. The effect of the time of addition of 8-azaguanine on the growth and recovery of motility by a non-motile culture of *Salmonella typhimurium*. -●—●- = control; -○—○- = 8-azaguanine (0·001 M) added immediately after transfer from 44° → 37°; ×—×- = 8-azaguanine (0·001 M) added 30 min. after transfer; -△—△- = 8-azaguanine (0·001 M) added 60 min. after transfer; -□—□- = 8-azaguanine (0·001 M) added 90 min. after transfer. Numbers denote % motile bacteria.

stained preparations showed a good correlation between the % flagellate bacteria and the % motile bacteria. If the flagella arise from synthesizing systems present in the bacteria then the effects of the analogues might be explained by assuming that the synthesis but not the functioning of these systems is sensitive to inhibition by 8-azaguanine and 2-thiouracil.

During logarithmic growth the mean no. flagella/bacterium and, by implication, the number of synthesizing systems remains approximately constant; there was a decrease during the stationary phase, probably due to cell division in the absence of growth. Addition of either 2-thiouracil (0·001 M) or 8-azaguanine (0·001M) to a culture of *Salmonella typhimurium* growing logarithmically at 37° resulted in an immediate decrease in the growth rate (Fig. 7). There was little change in the mean no. flagella/bacterium for 2 hr. after the addition of the inhibitor. Then the no. flagella/bacterium decreased and after 5 hr. was approximately half that of the control culture. The bacteria were still motile at the end of incubation, 2-thiouracil and 8-azaguanine apparently having had no effect on the functioning of preformed synthesizing systems. It would appear from these data that 2-thiouracil and 8-azaguanine inhibit the synthesis of functional synthesizing systems but do not completely inhibit growth and cell division, so leading to the decrease in the no. flagella/bacterium. Incubation of a non-motile culture of *S. typhimurium* for 2 hr. at 44° with 8-azaguanine (0·001 M) had no effect on the growth rate or the recovery of motility at 37° after the removal of the inhibitor.

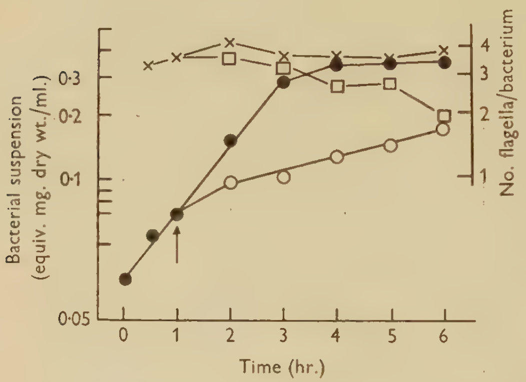

Fig. 7

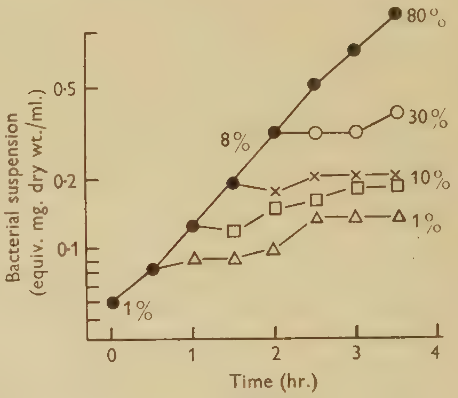

Fig. 8

Fig. 7. The effect of 8-azaguanine on growth of *Salmonella typhimurium* in a Casamino-acids + salts medium at 37° and on no. flagella/bacterium. -●—●- = growth, control culture; -○—○- = growth + 8-azaguanine (0·001 M); ×—×- = no. flagella/bacterium, control culture; -□—□- = no. flagella/bacterium, in presence of 8-azaguanine (0·001 M).

Fig. 8. The effect of 6-azauracil ($4{\cdot}3 \times 10^{-4}$ M) on growth and recovery of motility at 37° by a non-motile culture of *Salmonella typhimurium*. -●—●- = control; △—△- = 6-azauracil added 30 min. after transferring the culture from 44° → 37°; -□—□- = 6-azauracil added 60 min. after transfer; ×—×- = 6-azauracil added 90 min. after transfer; -○—○- = 6-azauracil added 120 min. after transfer. The numbers denote % motile bacteria.

Since none of the purine and pyrimidine analogues tested completely inhibited the regeneration of flagella by mechanically deflagellated *Salmonella typhimurium* it was possible to study the effects of these analogues on the formation of the synthesizing system. Inhibition by 6-azauracil did not produce such clear-cut results as did 2-thiouracil or 8-azaguanine (Fig. 8). Bacterial lysis resulted from the addition of 5-fluorouracil ($3·8 \times 10^{-4}$M) or FDUR (10^{-4}M) to a growing culture of *S. typhimurium*. 5-Fluorouracil ($3·8 \times 10^{-4}$M) inhibited the formation of the synthesizing system by *S. typhimurium* during incubation at 37° after growth at 44°, a result similar to that obtained with 8-azaguanine. Addition of 5-fluorouracil after incubation for 90 min. at 37° caused a decrease in the turbidity of the suspension due to bacterial lysis. Microscopic examination, however, showed that although only 5 % of the bacteria were motile at the time of addition, after incubation for 90 min. this had increased to 60 % (Fig. 9). FDUR (10^{-4}M) completely inhibited growth of *S. typhimurium* at 37° but at this concentration did not completely inhibit the recovery of motility, and after incubation for 3 hr. *c.* 15 % of the bacteria were flagellate (Fig. 10).

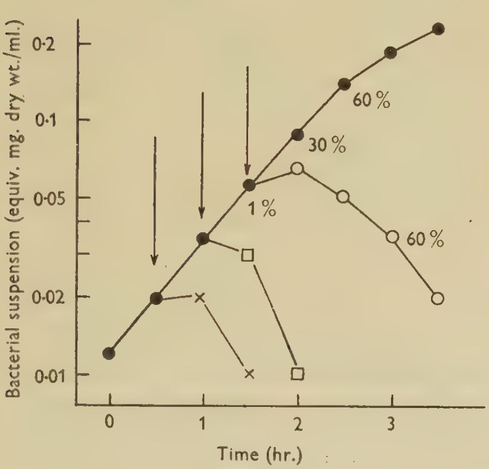

Fig. 9

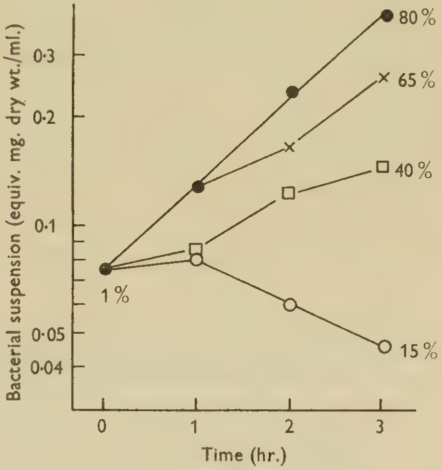

Fig. 10

Fig. 9. The effect of 5-fluorouracil ($3·8 \times 10^{-4}$M) on the growth and recovery of motility at 37° by a non-motile culture of *Salmonella typhimurium*. -●—●- = control; -×—×- = 5-fluorouracil added 30 min. after transfer from 44° → 37°; -□—□- = 5-fluorouracil added 60 min. after transfer; -○—○- = 5-fluorouracil added 90 min. after transfer. The numbers denote % motile bacteria.

Fig. 10. The effect of fluorodeoxyuridine (FDUR) on growth and recovery of motility by a non-motile culture of *Salmonella typhimurium*. -●—●- = control; -×—×- = $+10^{-6}$M FDUR; -□—□- = $+10^{-5}$M FDUR; -○—○- = $+10^{-4}$M FDUR. The numbers denote % motile bacteria.

Amino acid analogues. The growth of *Salmonella typhimurium* in a defined medium was affected by a number of amino acid analogues; but apart from *p*-FP none of the analogues tested inhibited the regeneration of functional flagella after mechanical removal. Addition of *p*-FP or *β*-2-thienylalanine (0·005 M) to cultures of *S. typhimurium* after growth at 44° prevented recovery of motility on incubation at 37°. The data for *p*-FP are shown in Fig. 11; *β*-2-thienylalanine gave essentially similar results.

Nitrogen mustard. Under suitable conditions nitrogen mustard will specifically inhibit DNA synthesis in micro-organisms, without completely inhibiting synthesis of RNA and protein. Pretreatment of deflagellated suspensions of *Salmonella* *typhimurium* with nitrogen mustard resulted in inhibition of regeneration of flagella during subsequent incubation in a complete medium (Table 3). Recovery of motility by *S. typhimurium* after growth at 44° was also inhibited by nitrogen mustard. Samples (10 ml.) were taken from a culture of *S. typhimurium* growing logarithmically at 44°, and incubated with different concentrations of nitrogen mustard for 10 min. at room temperature. The bacteria were harvested by centrifugation and resuspended in 20 ml. Casamino acid + salts + glucose medium and incubated at 37°.

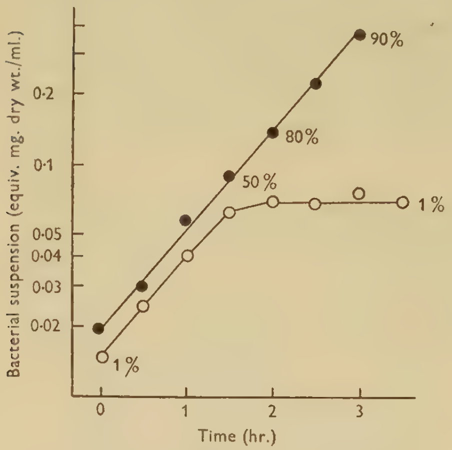

Fig. 11

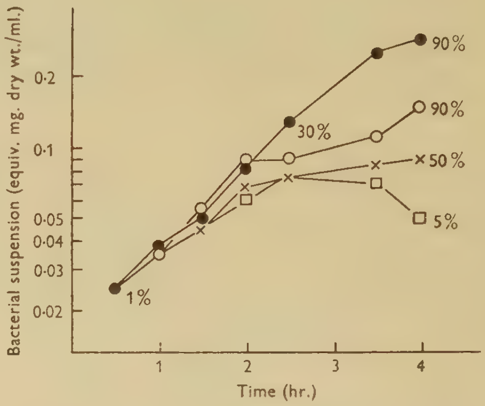

Fig. 12

Fig. 11. The effect of *p*-fluorophenylalanine on growth and recovery of motility at 37° by a non-motile culture of *Salmonella typhimurium*. -●—●- = control; -○—○- = + *p*-FP (0·005 M). The numbers denote % motile bacteria.

Fig. 12. The effect of pretreatment with nitrogen mustard on growth and recovery of motility by a non-motile culture of *Salmonella typhimurium*. -●—●- = control; -○—○- = + 100 μg. nitrogen mustard/ml.; -×—×- = + 250 μg. nitrogen mustard/ml.; -□—□- = + 500 μg. nitrogen mustard /ml. The numbers denote % motile bacteria.

Bacterial growth and motility were followed during the incubation at 37° (Fig. 12). Treatment with 100 μg. nitrogen mustard/ml. had little effect on the increase in optical density of the culture during the first 90 min. of incubation at 37°; after this there was some decrease in the rate of increase. Recovery of motility was similar to that of the control culture. With higher concentrations of nitrogen mustard there was some diminution in the initial increase in optical density at 37° and a progressive loss of ability to form flagella. Long forms were produced during incubation at 37°; some of these were motile and microscopic examination after flagellar staining showed that these forms possessed large numbers of flagella, suggesting that although cell division was inhibited, the synthesis of the synthesizing system and flagella occurred.

DISCUSSION

Cytological studies on motile bacteria have shown that in many cases the flagella originate from basal granules within the bacterial cell. These granules (blepharoplasts) have been clearly demonstrated in autolysed preparations from *Vibrio metchnikovi*, *Proteus vulgaris*, *Chromobacterium violaceum* (van Iterson, 1953), *V. cholerae*, *Rhodospirillum rubrum* (Grace, 1954), and *V. comma* (Tawara, 1957).

With the exception of *R. rubrum*, where several flagella are attached to one basal granule, each flagellum arises from a single basal granule. Houwink (1953) demonstrated hook-like bends at the base of each flagellum in a *Spirillum* sp. and van Iterson (1953) found similar 'hooks' at the ends of flagella torn out from *Proteus vulgaris*. Basal granules have not yet been demonstrated in *Salmonella typhimurium*, but it is a reasonable assumption that the flagella of this organism originate from subcellular structures ('basal granules') responsible for flagella synthesis and function.

The phenotypic expression of the ability to produce flagella is affected by the environment. For a number of bacterial species including *Pasteurella pseudotuberculosis* (Preston & Maitland, 1952), *Salmonella typhimurium* (Quadling & Stocker, 1956) and *Bacillus megaterium* (Vennes & Gerhardt, 1959) an increase in growth temperature results in the production of non-motile organisms although growth is unaffected. The transfer of a logarithmically growing culture of *S. typhimurium* from 37° to 44° results in a decrease in mean no. flagella/bacterium without affecting the growth rate, and on continued incubation at 44° the motile bacteria are diluted out. As regeneration of flagella can occur at 44°, it seems unlikely that the loss of motility was due to an inhibition of flagellar synthesis itself. Subsequent transfer of a non-motile culture from 44° to 37° results in the recovery of motility after a lag period equal to the mean generation time of the organisms, suggesting that a temperature-sensitive reaction is involved in the formation of the flagellum-synthesizing system.

None of the amino acid, purine or pyrimidine analogues tested, with the exception of *p*-FP, completely inhibited the regeneration of functional flagella by *Salmonella typhimurium*. Flagella synthesized in the presence of *p*-FP were non-functional and morphologically abnormal; the flagellar wavelength was approximately half that of normal flagella, $1{\cdot}04\,\mu \pm 0{\cdot}14\,\mu$ (S.D.) as compared with $2{\cdot}08\,\mu \pm 0{\cdot}1\,\mu$. The occurrence of two distinct flagellar wavelengths has been reported for a number of bacterial species; Pijper (1955) proposed the term 'biplicity' for this phenomenon. Unlike the changes in flagellar wavelength induced by variation in the pH value of the medium, the change in wavelength due to synthesis in the presence of *p*-FP was not reversible.

Data from experiments of the effects of inhibitors on the recovery of motility support the hypothesis that the 'basal granules' are absent from *Salmonella typhimurium* grown at 44° and are resynthesized at 37° during the lag before the appearance of flagella. Purine and pyrimidine analogues did not inhibit regeneration of flagella at 37° but prevented recovery of motility by *S. typhimurium* at 37° after growth at 44°. The apparent discrepancy between these data was resolved by the results of studying the effect of the time of addition of the analogue on the subsequent formation of flagella. Addition of 8-azaguanine (0·001 M), immediately after transferring a non-motile culture of *S. typhimurium* from 44° to 37°, resulted in the complete inhibition of formation of flagella; but when the addition of the analogue was delayed for 1 hr., formation of flagella was not affected, although at the time of addition only 5 % of the bacteria were flagellate. The formation of 'basal granules' in cultures growing logarithmically at 37° was also inhibited by 8-azaguanine and 2-thiouracil. Growth of the bacteria, although decreased, was not completely prevented, and there was a progressive decrease in the no. flagella/bacterium.

This effect is somewhat like that of temperature on formation of flagella, although in the latter case growth was not affected.

Since the addition of 8-azaguanine to logarithmically growing cultures of *Salmonella typhimurium* at 44° did not prevent the subsequent formation of flagella at 37° after the removal of the analogue, this suggests that macromolecular precursors of the 'basal granules' are not present at 44° but are synthesized *de novo* on transfer to 37°.

It is tempting to speculate on the nature of the 'basal granule' in *Salmonella typhimurium.* As flagella consist only of protein it is a logical assumption that the formation of the 'basal granules' will involve synthesis of ribonucleoprotein. The inhibition of the synthesis of the 'basal granules' by purine, pyrimidine or amino acid analogues would then be explicable, by assuming that the incorporation of these compounds results in the formation of an inactive ribonucleoprotein complex. The fact that these compounds have no effect on regeneration of flagella at 37° suggests that although synthesis of an active ribonucleoprotein complex may be essential for the formation of a functional 'basal granule', the further synthesis of a functional flagellum does not require concomitant synthesis of a biologically active RNA.

There is a marked parallelism between the data on the formation of bacterial flagella by *Salmonella typhimurium* and those obtained by Jeener, Hamers-Casterman & Mairesse (1959) on bacteriophage synthesis by a lysogenic strain of *Bacillus megaterium.* The synthesis of the phage was more sensitive to inhibition by 8-azaguanine and 2-thiouracil than was the formation of general cell protein. The data are consistent with the hypothesis that phage synthesis requires the prior formation of a particulate RNA fraction. Addition of 2-thiouracil or 8-azaguanine might be expected to result in much greater structural changes in the RNA required for phage synthesis, than in the RNA which determines the specificity of bacterial proteins, so accounting for the selective effect of these analogues.

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Interrelationship between Colicin Sensitivity and Phage Resistance

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SUMMARY

When cultures of *Escherichia coli* strain 15 were irradiated with ultraviolet radiation, the bacteria were induced to form a bactericidal material, colicin-15. Colicin-15 was released by the lysis of bacteria which underwent a residual growth without division. The only strains of bacteria sensitive to the action of colicin-15 were derivatives of strain 15. Three colicin-resistant strains were obtained, all of which became simultaneously sensitive to every T-phage, while the colicin-sensitive parental strains were lysed only by T2. By stepwise reversal of the phage sensitivity pattern, it was possible to revert colicin-resistant mutants to colicin sensitivity again. Only certain mutations conferring resistance in concert to phages T1, T3, T4, T5 and T7 seemed to result in colicin sensitivity. A model which will account for these phenomena is presented.

INTRODUCTION

Certain strains of bacteria possess the inherent capacity to produce specific substances which have lethal effects on other related bacterial strains. The antagonism between two strains of *Escherichia coli* was first described by Gratia (1925), who found that the filtrate from a culture of *E. coli* V greatly inhibited the growth of *E. coli* ϕ. It was later shown that strain V produced an antibiotic substance active against strain ϕ (Gratia, 1932). The frequent occurrence of such reciprocal antibiotic action among many strains of *E. coli* was demonstrated by Fredericq (1946*a*), and the substances responsible for the lethal effects were named 'colicins'. Further investigations of numerous enteric bacteria revealed that similar substances were also formed by some strains of *E. freundii* (Fredericq, 1947), a few Salmonella strains (Fredericq, 1952; Hamon, 1955), and many Shigella strains (Fredericq, 1948*a*; Fastier, 1949). Moreover, analogous substances were found in three other families of bacteria: pyocin, produced by *Pseudomonas pyocyanea* (*aeruginosa*) (Jacob, 1954); megacin, produced by *Bacillus megaterium* (Ivanovics & Alföldi, 1954); and pesticin, produced by *Pasteurella pestis* (Ben-Gurion & Hertman, 1958). A more general term 'bacteriocin' was proposed (Jacob, Lwoff, Siminovitch & Wollman, 1953) for these substances, since the name colicin was originally intended to characterize the antibiotic principle from *E. coli*.

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Colicins differ in the extent and specificity of their activity, and in their physical and chemical properties (Fredericq, 1948*b*; Goebel, Barry & Shedlovsky, 1956; Hamon, 1956; Ludford & Lederer, 1953). Usually, a colicinogenic strain of bacteria spontaneously produces a specific colicin which is effective against another strain. Under conditions similar to those which cause induction of temperate phage in lysogenic bacteria, Jacob, Siminowitch & Wollman (1952) showed that it was possible to induce the formation of colicin by ultraviolet (u.v.) irradiation. The genetic capacity to form colicins resides in transmissible units, procolicins, which behave much as do prophage in lysogenic bacteria. Ryan, Fried & Mukai (1955) reported the existence of a u.v.-inducible colicinogenic strain which was susceptible to the colicin it produced. The present paper describes the induction of colicin synthesis in this strain, and the specificity of action of the colicin produced.

METHODS

Bacteria. The thymine- and histidine-requiring strains of *Escherichia coli* used in these experiments were originally secured after X-ray treatment of the wild type strain 15, American Type Culture Collection No. 9723 (Roepke, Libby & Small, 1944). The author is grateful to Dr S. B. Greer for the thymineless strain, hereafter designated t-CS, and to Dr F. J. Ryan for the histidineless strain, h-CS. The notation 'CS' is used to signify colicin sensitivity. All stock cultures were maintained at 5° on nutrient agar slopes.

Phages. The seven phages of the T-series were used (Delbrück, 1946). High titre phage stocks (10^9–10^{11} particles/ml.) were prepared by infecting growing cultures of *Escherichia coli* B with each of the seven phages. Following mass lysis, the cultures were centrifuged at low speed to remove bacterial debris, and the supernatant fluids shaken with chloroform to kill remaining viable bacteria. The stock suspensions thus obtained were stored at 5° in screw-cap test tubes.

Media. For all colicin experiments a modified Gray & Tatum's defined medium (Ryan & Schneider, 1949), supplemented with 0·06% (w/v) glucose, and either 2 μg. thymine/ml. or 25 μg. histidine/ml. was used. Agar medium was prepared by adding 20 g agar/l. liquid medium and increasing the concentration of glucose to 0·5% (w/v).

For phage experiments, nutrient medium consisting of 8 g./l. desiccated Difco nutrient broth + 0·5% (w/v) NaCl was used (Adams, 1950). For plating, this nutrient medium was solidified with 1·5% (w/v) agar.

U.v.-irradiation. U.v.-irradiations were performed with a 3·5 W. Westinghouse Sterilamp WL 793, emitting predominantly at 2537 A. For the irradiation, one ml. of a stationary phase culture of *Escherichia coli* strain t-CS or h-CS was inoculated into 100 ml. defined medium, giving a suspension containing *c.* 5×10^6 bacteria/ml. After 4 to 5 hr. of aerated growth at 37°, when the bacteria were in the logarithmic phase of growth and at a concentration of 4–8×10^7 organisms/ml, 15 ml. samples were distributed into Petri dishes and exposed to various doses of u.v. radiation. After irradiation, duplicate 6 ml. samples were pipetted into 16 × 150 mm. test tubes and incubated at 37° until maximal clearing. The growth of the cultures was measured turbidometrically in a Klett–Summerson colorimeter fitted with a no. 59 filter. For unirradiated cultures there was a linear relationship between total number of bacteria/ml. and the colorimeter reading as found for this strain by Ryan & Schneider (1948). After maximal clearing, 0·2 ml. chloroform was added to one of each pair of tubes which were then shaken vigorously for 15 sec. and centrifuged; the supernatant fluids were saved for subsequent testing of bactericidal activity. The growth in the second group of tubes was followed until the cultures reached a stationary phase due to the exhaustion of glucose. The supernatant fluids of these tubes were kept as before. All operations during and after irradiation were conducted under yellow light to prevent photoreversal of the u.v. effects (Jacob *et al.* 1952).

Demonstration of bactericidal activity. Approximately 5×10^7 organisms (unirradiated strain t-CS or h-CS) were spread on the surface of defined medium agar plates which had been previously dried at 37° for 2 days. Measured samples, usually 0·05 ml. of the supernatant fluids obtained following u.v. induction, were placed on the seeded plates. The plates were kept at room temperature until the drops had dried, and were then incubated at 37° for 16 hr. Antibacterial activity was manifested by the fact that, except for a few isolated colonies, no growth occurred in the area covered by the drops. Cell-free supernatant fluids from unirradiated cultures were used as controls. The small colonies within the clear areas consisted of bacteria which did not grow when transferred to nutrient agar slopes. When a twofold dilution was made of active lysates, the clarity of the spots faded markedly, and no unequivocal estimate of colicin titre could be made. Therefore, only undiluted supernatant fluids were used to test for antibacterial activity.

Isolation of phage-resistant mutants. Approximately 10^9 organisms from a broth-grown culture of the phage-sensitive strains were spread on the surface of nutrient agar plates. Duplicate plates were spotted with 0·1 ml. of high titre stock suspension or the different phages, with no more than three spots to a plate. The drops were allowed to spread by tilting the plates, care being taken to prevent merging and mixing of the phages. After the drops had dried, the plates were incubated at 37° for 16–24 hr. before observation and isolation of colonies of presumably resistant bacteria. These colonies were stabbed with a sterile needle, inoculated into 2 ml. of nutrient medium, and incubated for 6–8 hr. until a majority of the tubes became turbid. Whenever possible, at least five isolates were made for each viral strain. A preliminary test of resistance was made at this time by the cross-streaking method of Demerec & Fano (1945). Suspensions of phage and bacteria were streaked at right angles on a nutrient agar plate. An isolate was classified as resistant to a phage when it showed no lysis at the intersection of the streaks. Such mutants were then purified by streaking on nutrient agar plates and making three successive isolations of single colonies. To make certain that mutants to be kept for further study had stemmed from independent mutations, only one isolate from a group was maintained unless some members of the group showed either a different cross-resistance pattern, or a recognizable difference in colony morphology.

The notation of bacterial strains derived from phage-resistant colonies follows the system of Demerec & Fano (1945). Thus, t-CR/5,1 indicates that the resistant strain was derived from t-CR after selecting with T5, and found to be cross-resistant to T1.

RESULTS

Induction of colicin synthesis

An example of the behaviour of *Escherichia coli* strain t-CS cultures following exposure to various doses of u.v. radiation is shown in Fig. 1. After an initial increase in optical density lasting about 90 min., a decrease in turbidity occurred. The extent of the initial increase diminished as the dose increased. Subsequently, a second increase in optical density at similar rates occurred and eventually reached a stable value. Bactericidal activity was present in the supernatant fluids of all of the irradiated cultures, taken either at the time of maximal clearing or after the stationary phase of growth was reached. Qualitatively, the activity was highest in the supernatant fluids of the cultures which showed maximal growth before lysis. No activity was found in the unirradiated controls.

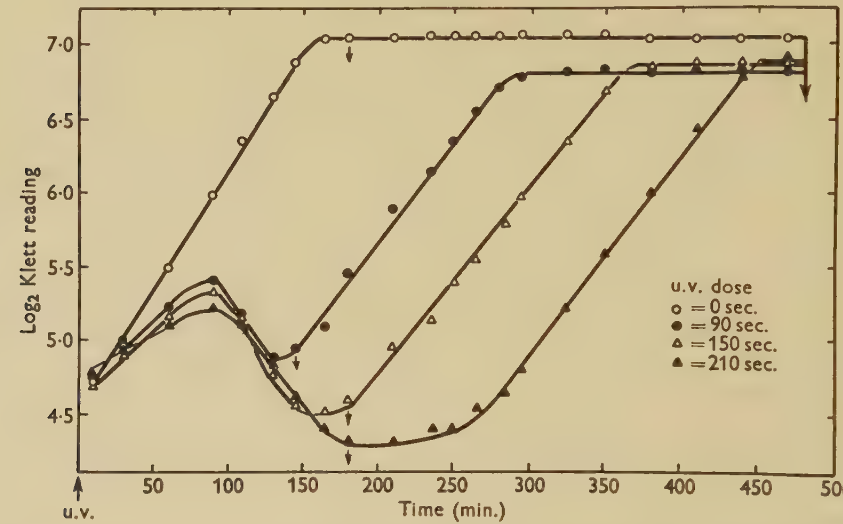

Fig. 1. Growth after u.v.-irradiation of cultures of *Escherichia coli*, t-CS, in defined medium. Arrows indicate times at which cultures were centrifuged to obtain supernatant fluids.

The initial increase in optical density was not the result of the division of those bacteria which survived the irradiation. This was determined by plating for surviving bacteria at intervals following induction (Table 1). Since coli strain t-CS is sensitive to the colicin it produces, a colicin-resistant mutant derived from coli strain t-CS was used as a control. An inducing dose of u.v. radiation which gave a maximal post-irradiation rise in turbidity was used. Rather than an increase in colony formers associated with the increase in optical density, a gradual decrease was observed with both strains. Bactericidal activity was found in the supernatant fluids only after the cultures showed a decrease in turbidity.

Microscopic observation of the induced cultures revealed the following changes in morphology of the bacteria: during the initial period after irradiation, *c.* 90 % of the organisms gradually elongated and became less motile. The elongation proceeded uniformly, and after *c.* 100 min. the bacteria were two to three times their original length. There was no apparent increase in the total number of bacteria. While actual lysis was not observed, there was a sudden decrease in the number of elongated bacteria concomitant with the decrease in optical density. The second rise in Klett readings was correlated with the increase of regular-sized rod-shaped bacteria which increased in number with time.

On the basis of these experiments, it is concluded that the residual growth without division of induced cultures represented a latent period during which colicin was being synthesized in those bacteria which elongated, and that the lysis of these bacteria released the colicin into the culture medium.

Table 1. *Comparison between optical density and the number of viable bacteria after u.v. induction* (90 *sec. dose*) *of* Escherichia coli *strain t-CS and t-CR. The number of viable bacteria was determined by plating on defined medium agar supplemented with* 2 *μg. thymine/ml.*

Time after u.v. irradiation (min.)	Strain t-CS		Strain t-CR	
	Optical density (Klett units)	No. of viable bacteria/ml.	Optical density (Klett units)	No. of viable bacteria/ml.
0	54	$9{\cdot}7 \times 10^6$	58	$9{\cdot}6 \times 10^6$
20	58	$1{\cdot}6 \times 10^6$	66	$1{\cdot}9 \times 10^6$
40	65	$1{\cdot}2 \times 10^6$	73	$1{\cdot}7 \times 10^6$
60	72	$6{\cdot}4 \times 10^5$	80	$1{\cdot}1 \times 10^5$
80	74	$7{\cdot}7 \times 10^5$	79	$7{\cdot}0 \times 10^5$
100	67	$1{\cdot}1 \times 10^6$	62	$1{\cdot}0 \times 10^6$
120	53	$1{\cdot}1 \times 10^6$	47	$1{\cdot}1 \times 10^6$

Post-irradiation death of the sort described above was reported by Lieb (1951). She suggested that two types of survivors of u.v. irradiation were present immediately after irradiation: (i) 'liquid resistants' which divided at normal rates in liquid medium; (ii) 'liquid sensitives' which died when placed in liquid medium, but which survived when plated immediately. In the present experiments, it is not known whether or not the bacteria which died when left in liquid medium were included among the bacteria which elongated and produced colicin.

Transitory and heritable resistance to colicin-15

Since bactericidal activity was found in the supernatant fluids of induced cultures which had reached a stationary value of optical density, it was presumed that the bacteria present were colicin-resistant. One possibility for the presence of such resistant organisms would be that the stock culture of coli strain t-CS was heterogeneous, containing some resistant and some sensitive bacteria. However, when 15 single-colony isolates were made from coli strain t-CS, each isolate showed the same behaviour as the parental culture, producing the same colicin in response to induction which was active against itself and the parent. Moreover, when induced cultures which had reached a stationary value were centrifuged and the organisms used as indicator bacteria, they proved to be equally sensitive to colicin-15 as were organisms of unirradiated cultures. The isolation and test of clones from 20 such organisms confirmed this result.

Further evidence for the transitory resistance indicated above was shown by another method of testing for colicin activity; this consisted of plating for viable bacteria following exposure to colicin (Jacob *et al.* 1952). Tenfold dilutions were made of a stationary phase culture of coli strain t-CS and 0·1 ml. samples of these dilutions were suspended in 0·9 ml. of an active sample of colicin-15. After standing at room temperature for 30 min., appropriate dilutions were plated for viable bacteria. Colonies were counted after incubation for 2 days at 37°. The results of such platings are shown in Table 2. Regardless of the initial number of bacteria, from 22 to 39 % survived. When ten survivors from each dilution were isolated and tested for sensitivity, all forty isolates proved to be sensitive. The survival of organisms which grow after exposure to active lysates must be explained as a result of some sort of transitory resistance, the nature of which is unknown.

Nevertheless, two different mutants of coli strain 15 were secured which possessed a heritable resistance to colicin-15, one from coli strain t-CS and the other from coli strain h-CS, designated t-CR and h-CR, respectively. These mutants were found as viable colonies which grew within the clear areas during routine testing of colicin activity. Besides being heritably resistant to colicin-15, both mutants were sensitive to all seven T-phages. Both parental coli strains h-CS and t-CS, on the other hand, were sensitive only to phage T2; this sensitivity could be clearly shown by the cross-streak method only when tryptophan (2 mg./ml.) was present. On the contrary, the CR coli strains sensitive to all seven phages showed a clear reaction to phage T2 in the absence of tryptophan. A change in sensitivity to T-phages was first reported by Ryan (1953) in his study of orthoselection in the coli strain h-CS. The fitter-type mutant obtained was also found to be sensitive to the seven T-phages, and resistant to the colicin it produced. This curious change to phage sensitivity in three independent colicin-resistant mutants suggested the existence of a correlation between colicin sensitivity and phage resistance. It was of interest to determine whether changes of the phage-sensitive phenotype in colicin-resistant mutants would restore colicin sensitivity.

Table 2. *Survival of* Escherichia coli, *strain t-CS, exposed to colicin*-15. *The number of bacteria was determined by plating on defined medium agar supplemented with* 2 *μg. thymine/ml.*

Initial no. of bacteria	Final no. of bacteria	Surviving fraction (%)
$4{\cdot}1\times10^6$	$1{\cdot}1\times10^6$	27
$4{\cdot}0\times10^5$	$1{\cdot}3\times10^5$	33
$4{\cdot}0\times10^4$	$8{\cdot}6\times10^3$	22
$4{\cdot}9\times10^3$	$1{\cdot}8\times10^3$	39
	Average	30

Relationship between colicin sensitivity and phage resistance

When phage-resistant strains selected by each of the seven T-phages were checked for their cross-resistance pattern, a grouping similar to that obtained for *Escherichia coli* B (Demerec & Fano, 1945) was found; this is shown in Table 3. None of these single-step mutants was sensitive to colicin-15. Thus, resistance to one or a few phages did not result in a loss of colicin resistance.

Since the colicin-sensitive parental coli strains were sensitive only to phage T2, it was felt that colicin sensitivity might be restored only in strain having the same phage-resistance pattern. Therefore new mutants were obtained by selecting for additional mutations in these one-step mutants. The pedigrees of multiply-resistant strains obtained are represented for coli strains t-CR and h-CR in Figs. 2 and 3, respectively. Strains which became sensitive to colicin-15 were found only among those mutants that were resistant to at least phages T1, T3, T4, T5 and T7. For example, coli strain t-CR/5, 1/3, 7 became sensitive to colicin only after it became resistant to phage T4. The presence or absence of phage T6 resistance did not affect colicin sensitivity.

Table 3. *Types of phage-resistant strains derived from* Escherichia coli, *strains t-CR, and h-CR, after selection with the T phages*

Parent strain	Selecting phage	Mutant strains
t-CR	T1	t-CR/1, 5
	T2	t-CR/2
	T3	t-CR/3, 7; t-CR/3, 4, 7
	T4	t-CR/4, 3; t-CR/4, 3, 7
	T5	t-CR/5, 1
	T6	t-CR/6
	T7	t-CR/7, 3; t-CR/7, 3, 4
h-CR	T1	h-CR/1, 5
	T2	(none)
	T3	h-CR/3, 4; h-CR/4, 3, 7
	T4	h-CR/4; h-CR/4, 3; h-CR/4, 3, 7
	T5	h-CR/5, 1
	T6	h-CR/6
	T7	h-CR/7, 3; h-CR/7, 3, 4

On the other hand, resistance to the five essential phages did not automatically confer sensitivity to colicin. Thus colicin-resistant mutants were found which, in their reaction to these phages, were phenotypically similar to colicin-sensitive mutants. Such strains must be assumed to be genotypically dissimilar. The fact

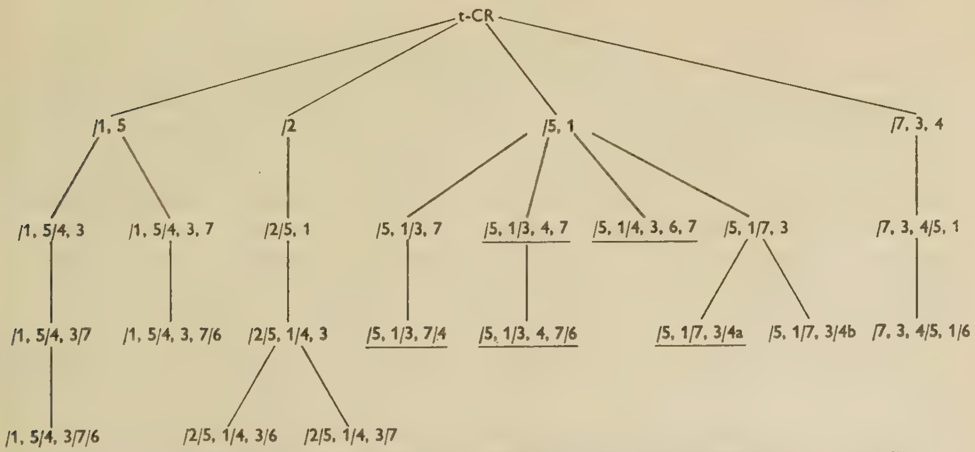

Fig. 2. The pedigree of phage-resistant strains derived from *Escherichia coli*, t-CR. For the sake of clarity, the notation for the parent strains has been omitted. Mutants sensitive to colicin-15 are underlined.

that no colicin-sensitive mutants could be obtained from coli strain h-CR (Fig. 3) must similarly be attributed to genotypic differences, either between coli strains h-CR and t-CR, or between the particular phage-resistant mutants derived therefrom. Indeed, it has been found that coli strain h-CR consistently showed a higher efficiency of plating with respect to phage T2 than did coli strain t-CR.

After transfer of the fertility agent, F^+, to auxotrophic mutants of *Escherichia coli* B De Haan (1954) obtained genetic data for this previously infertile strain. Similar attempts to infect coli strain 15 with F^+ were unsuccessful. Since sexual recombination does not occur, the assumption of genotypic dissimilarity could not be experimentally ascertained. However, in bacterial strains where genetic analysis is possible, phenotypically similar phage-resistant mutants have been shown to be genotypically dissimilar (Lederberg, 1947; De Haan, 1954). Also, resistance to a given colicin has been shown to be determined by more than one locus (Fredericq, 1958).

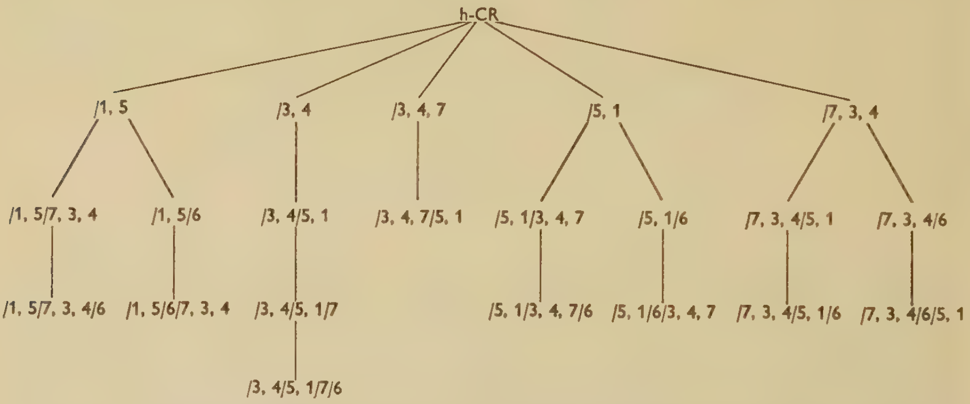

Fig. 3. The pedigree of phage-resistant strains derived from *Escherichia coli*, h-CR. For the sake of clarity, the notation for the parent strains has been omitted. No mutants were sensitive to colicin-15.

The nature of colicin-15

Before studies could be made on the properties of colicin-15, it was necessary to obtain a more sensitive indicator strain. Numerous strains of *Escherichia coli* other than strain 15, as well as *Shigella sonnei*, *Bacillus megaterium*, and *Salmonella typhimurium* were found to be resistant. The finding that colicin sensitivity in coli strain 15 was associated with resistance to phages T1, T3, T4, T5, and T7 suggested the necessity of securing another strain of *E. coli* with a similar phage-resistance phenotype. Strain B/1, 5/3, 4, 7 secured from *E. coli* B and strain K12/1, 5/3, 4, 7 secured from *E. coli* K12 were both resistant to colicin-15.

Fredericq (1957) demonstrated two different mechanisms of resistance to colicins: (i) resistance by loss of receptors; (ii) immunity by acquirement of ability to produce colicins. A strain possessing the receptor for colicin-E was made colicinogenic for colicin-E. The resulting strain became immune to this colicin while still keeping the corresponding receptor. Based on these findings, it was hoped that a non-colicinogenic mutant of coli strain 15 would retain its receptor for colicin-15. Attempts to secure such a mutant by u.v. irradiation (Ivanovics & Nagy, 1958) or with cobalt chloride and acriflavine (Hirota, 1956, 1957; Furness & Rowley, 1957) were unsuccessful. Also bacteria which survived heat treatment (Zamenhof & Greer, 1958) or which had passed through the protoplast state did not lose the colicinogenic property.

The inability to find a good indicator strain precluded extensive investigations of the chemical, physical and biological properties of colicin-15. However, with coli strain 15, the following facts were observed. (1) Storage at 5° for at least 1 year, or exposure to 8700 ergs of u.v. radiation/mm.^{2} did not result in a loss of bactericidal activity. (2) Heating at 70° for 30 min., or treatment with commercial trypsin for 10 hr. destroyed activity. (3) Filtration through a Millipore filter resulted in an appreciable loss of activity. (4) High speed centrifugation (20,000 *g*) for 30–60 min. did not seem to result in loss of activity, but after 120 min. there was no activity left in the supernatant fluid.

DISCUSSION

The induction of colicin-15 formation described here is analogous to the results of Jacob *et al.* (1952) for colicin ML and of Ivanovics & Alfödi (1955, 1957) for megacin. In all these cases, a bacteriocin is released into the culture medium after a latent period followed by mass lysis of the induced bacteria. However, some authors (Fredericq, 1954, 1955; Hamon & Lewe, 1955; Kellenberger & Kellenberger, 1956) have concluded that induction of bacteriocin formation does not result in lysis unless the induced strains are also lysogenic. For example, *Escherichia coli* ML was found to be lysogenic, and lysis in this case was attributed to the development of temperate phages. The u.v. irradiation of a colicinogenic but non-lysogenic strain resulted in a residual growth of the bacteria, followed by a stationary phase without apparent lysis during which time the production of colicin occurred. On the other hand, megacin formation was shown to be invariably associated with the lysis of the induced bacteria. Fifty-two non-megacinogenic mutants derived from megacinogenic parents could not be made to lyse by exposure to u.v. irradiation (Ivanovics & Nagy, 1958). Electron microscope studies of active lysates failed to demonstrate the presence of any phage particles, and it was concluded that the bacteriocinogenic character of *Bacillus megaterium* was not associated in the slightest degree with phage production (Ivanovics, Alfödi & Lovas, 1957).

We have been unable to detect the presence of any virus in active lysates of colicin-15, although numerous phage-sensitive strains were used as indicators. However, such a finding still does not rule out the possibility that coli strain 15 is lysogenic. Arber & Kellenberger (1958) made electron microscope examination of the lysates of seven defective-lysogenic strains derived from *Escherichia coli* K12 (λ). Although the lysates revealed no morphologically complete phages, each revealed the presence of phage-related structures: four of the strains produced empty heads and tails, while three produced heads only. All seven strains synthesized serum-blocking material (De Mars, 1955). Until similar studies are made, the possibility that our strains contain a defective prophage, the induction of which results in the synthesis of colicin-15, is not excluded.

Susceptibility to colicins has been shown to be controlled by a number of receptors, each specific for the colicin to which a strain is sensitive (Fredericq, 1946*b*). The existence of genetic factors controlling the presence or absence of the receptors was shown by recombination studies (Fredericq & Betz-Bareau, 1952). Usually, a mutation affecting the receptor for one colicin does not affect the receptors for other colicins nor the receptors for bacteriophages. However, in the case of colicin-K and phage T6, a specific cross-resistance was observed (Fredericq & Gratia, 1950). Since all resistant mutants selected by either colicin-K or phage T6 were resistant to both agents simultaneously, it was concluded that a single receptor was shared by the two agents. A similar relationship was found for colicin-E and phage BF23 (Fredericq, 1949).

The specificity of colicin-15 indicates that its activity also requires the presence of a specific receptor. In contrast to the reciprocal cross-resistance mentioned above, colicin-15 sensitivity shows an inverse relationship with phage-resistance. A formal scheme to explain this relationship is presented in Fig. 4. The diagram is a modification of the generalized scheme formulated by Anderson (1944), explaining interrelationships between phage-resistance and metabolic changes leading to increased growth requirements. The receptor site for colicin-15 is depicted as being composed of the receptor sites for phages T(1, 5), T3, T4 and T7. Since no mutants were found which were resistant to phage T1 or phage T5, alone, the receptor sites for these phages have been combined. Pathways I and II lead to the formation of elements which must be present to enable the bacteria to react with phages T(1, 5) and T3, T4 or T7, respectively. Mutational changes at a or b, would result in different alterations in R(1, 5), while changes at c, d, e, f, g, or h would result in different alterations in the respective receptor sites concerned. All of the phage-resistant phenotypes observed (Figs. 3, 4) can be accounted for by this scheme. Since some mutants with the correct phage-resistant phenotype remained colicin-resistant, it is necessary to require that a combination of precise mutant receptor sites is needed for colicin sensitivity. The possible role of the sequence in which the mutations occurred could not be elucidated.

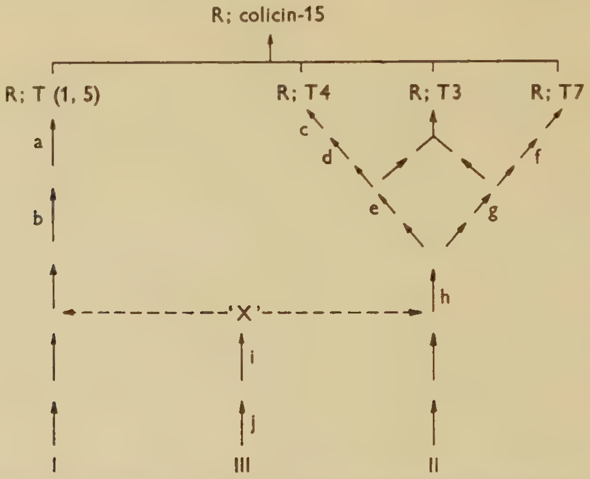

Fig. 4. Schematic representation of the relationship between colicin sensitivity and phage resistance. See text for explanation. *R* = receptor site for the adsorption of phage (T) and colicin-15. a, b, c, d, e, f, g, h, i, j, = mutations resulting in alteration of receptor site.

In order to explain the observation that a single change from colicin sensitivity to resistance (coli strain t-CS to strain t-CR and coli strain h-CS to strain h-CR) resulted in susceptibility to all phages, it is necessary to place pathways I and II under unit control. This can be done by a common factor 'X' formed by pathway III. The factor 'X' might represent an element common to all phage receptor sites. If this were so, the parental CS coli strains would be geno-typically resistant to all T-phages except T2, and the mutation (i or j) to strain CR would result in the observed sensitivity to the phages. In this case, it should have been possible to obtain a one-step mutant which became simultaneously phage-resistant and colicin-sensitive. Such a strain was not found. On the other hand, 'X' might be an element which exerted an epistatic effect on the phage receptor sites. In this event, the parental strains would be phenotypically phage-resistant but genotypically sensitive. The mutation to CR would result in the removal of 'X', allowing the expression of the phage-sensitive phenotype. Since reversal to colicin sensitivity required stepwise mutations to multiple phage-resistance, evidence exists for the second role of factor 'X'. However, in the absence of recombination data, we cannot conclusively distinguish between the two alternatives.

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The Fine Structure of Bacteriophages

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SUMMARY

The 'negative contrast' method for the preparation of viruses for electron microscopy was used to study 22 different bacteriophages. A great variety of features is described, leading to some morphological grouping. The use of uranyl acetate on coli phage T 5 revealed subunit structure in both the head and tail, features not shown by the conventional phosphotungstic acid treatment.

INTRODUCTION

Until the application of the 'negative contrast' technique to the electron microscopy of bacteriophage particles by Brenner *et al.* (1959), all published electron micrographs had been obtained of shadowed or, occasionally, of sectioned specimens. The smallest resolvable detail in shadowed specimens consists of the discrete crystallites of the shadowing material so that the fine structure of the phage particles themselves is obscured rather than revealed. On the other hand, 'negative contrast' methods are capable of making full use of the high resolving power (10 Å or less) of modern electron microscopes, thus permitting the examination of hitherto invisible details of bacteriophage structure. An examination of the literature shows that the most intensively studied bacteriophage from every point of view is the coli phage T 2 which was, incidentally, the subject of the highly informative work of Brenner *et al.* (1959). In comparison with coli phage T 2 little is known about other phages, particularly those outside the coli group. It was therefore considered of value to study a variety of phages now that higher resolution is available. The authors of the present paper have not attempted to examine phages active against all the bacterial species which possess them. A collection of 22 different phages is described; they are active on the following bacteria: the coli-typhoid group, Staphylococcus, *Streptococcus lactis*, *Pseudomonas aeruginosa* and *Brucella abortus*. A wide variety of structures has been observed but certain clear groupings into morphological types can be made.

METHODS

Bacteriophages used

Coli-typhoid phages. The three phages of the coli T group (of which there are seven altogether), T 1, T 3 and T 5, were obtained some years ago from Professor T. F. Anderson. Stocks for electron microscopy were prepared on *Escherichia coli* B growing in a defined medium supplemented with 0·00025 M-calcium chloride in the case of T1 and T5 (Kay, 1959). Phages 11F, C4, S1BL, S6BL, 66F, 3T+ were isolated from samples of raw and effluent sewage, kindly supplied by the Oxford sewage works, by growth and selection of phages on certain coli and typhoid bacteria (Fildes & Kay, 1957). The unadapted Vi phage no. 1 was obtained some time ago from the late Dr A. Felix. Typhoid phages 1 and 2 were described by Fildes & Kay (1955).

Staphylococcus phages. Six of the Staphylococcus typing phages were kindly supplied by Dr R. E. O. Williams (Central Public Health Laboratories, Colindale, London, N.W. 9). They were numbered 3B, 6, 52, 71, 77 and 187. The suspensions were used as supplied in broth. No attempt was made to grow more of these phages.

Streptococcus lactis *phage.* This was kindly supplied by Mr B. Reiter (National Institute for Research in Dairying, Shinfield, Reading).

Pseudomonas aeruginosa *phages.* We are indebted to Dr Lois Dickinson (Boots Pure Drug Co. Ltd., Nottingham) for a supply of three phages, Pa, Pc and Pz. Pa and Pz are lysogenic phages and Pc is a virulent mutant isolated from Pz. Stocks of these phages were prepared on a strain of *P. aeruginosa* C10 also supplied by Dr Dickinson.

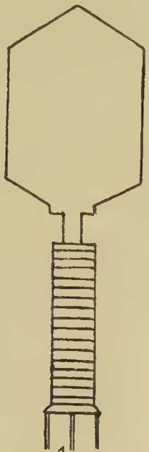
Brucella phage. This phage was supplied by Dr W. J. Brinley Morgan (Veterinary Research Station, Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey) as a suspension in broth. It is active on a strain of *Brucella abortus* and is referred to in greater detail by Brinley Morgan, Kay & Bradley (1960).

Preparation of specimens for the electron microscope

The technique used was essentially that of Brenner *et al.* (1959), though the use of potassium phosphomolybdate and uranyl acetate was suggested by Nixon (1960). The principle is that the phage particles are embedded in a dried microdroplet of electron-dense material on a suitable support film, and examined directly in the electron microscope. The details of the method as used are as follows.

The phage suspension in nutrient broth or in phosphate buffer (0·033 M, pH 7·6) was first centrifuged at low speed to remove bacterial debris. The phage particles themselves were then sedimented at about 8000 *g* and the supernatant fluid discarded. The pellet was resuspended in a solution of potassium phosphotungstate or phosphomolybdate prepared by adjusting a 1 % (w/v) solution of the appropriate acid with potassium hydroxide to pH 7·2. An electron microscope specimen support grid was coated with a carbon film containing small holes as an aid to focusing, washed in chloroform to remove grease, and then touched on to the surface of the phage suspension so that it was covered with a thin film of liquid. After drying in air the specimen was ready for examination.

In the case of the calcium-dependent phage T5, uranyl acetate was used instead of potassium phosphotungstate or phosphomolybdate. A 1 % (w/v) solution of uranyl acetate in water is at *c.* pH 3·5, and this pH value cannot be increased without causing a precipitate. It was therefore necessary to use the solution of this low pH value for the final embedding. To decrease the chance of osmotic effects the phage was transferred through an intermediate solution of ammonium acetate (pH 5·0, 0·033 M) from the phosphate buffer, by centrifugation, before finally suspending in the uranyl acetate solution. This procedure also removed traces of phosphate which would have precipitated uranium. Both the ammonium acetate and the uranyl acetate contained 0·0005 M-calcium chloride in an attempt to decrease the inactivation of the phage.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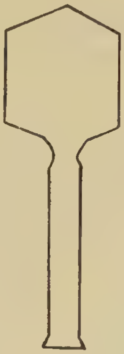
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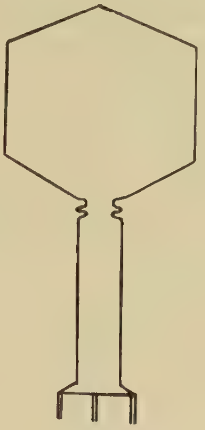
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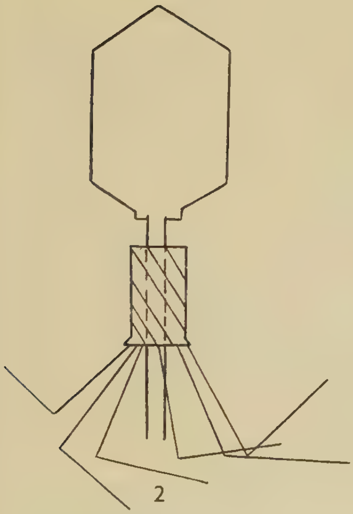
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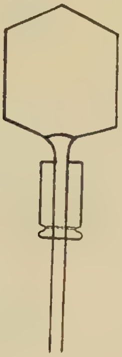
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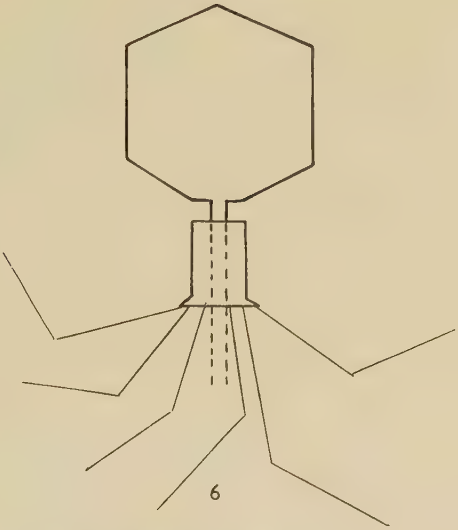
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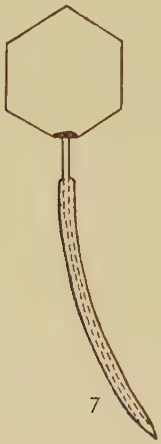
Fig. 1. T2 type, active.
Fig. 2. T2 type, inactive.
Fig. 3. Phage 2, active.
Fig. 4. Phage 2, inactive.
Fig. 5. Phage Vi1, active.
Fig. 6. Phage Vi1, inactive.

Description of the phage particle structures

It is generally agreed that all phages so far examined contain deoxyribonucleic acid (DNA) and that this material, which may amount to 50 % of the weight of the phage, is contained in the head of the particle. DNA as such cannot be recognized in electron micrographs of phage, but its presence or absence in the phage head can be observed with some certainty. By using the 'negative contrast' technique, the phage particles appear as electron-transparent objects set in an electron-dense surround which contains the phosphotungstate. This indicates that the phosphotungstate is not absorbed by the particles and thus a considerable contrast difference is obtained. Should the phage particle have lost its DNA, this will be replaced in the head by phosphotungstate which diffuses through the head membrane. Thus the contrast provided by an empty head will be much less than that provided by one full of DNA. It is therefore possible to distinguish between intact and changed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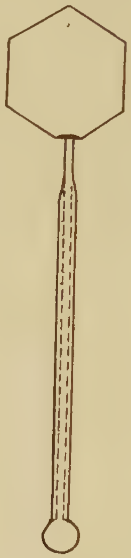
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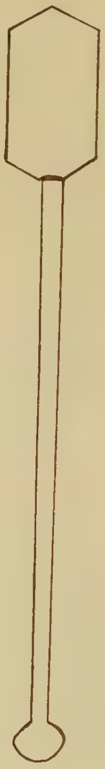
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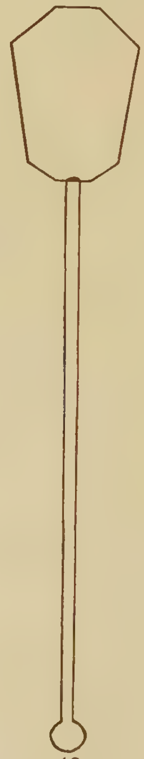
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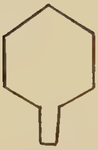
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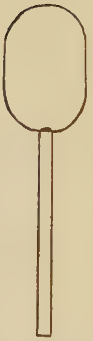
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Fig. 7. SBL group, phage 1, phages T1 and T5; general appearance only; the dimensions vary.
Fig. 8. Phages Pc, 187, 77, etc., general appearance only; the dimensions vary.
Fig. 9. Phage 6.
Fig. 10. Phages T3 and Brucella phage.
Fig. 11. Phage 3ML.
Fig. 12. Phage 3B.

particles in the electron microscope. It seems likely that the changed particles correspond in appearance to those which have injected their DNA into a host cell.

The electron micrographs of the phages clearly show the morphological details, but for clarity the more important features are illustrated in the line drawings (Figs. 1–12).

Table 1. *Dimensions of phage particles*

Phage	Head* (Å)	Tail length (Å)	Sheath width† (Å)	Core width (Å)
Coli T2‡	1000 × 700 (950 × 650)§	1100 (periodicity 30) (1000)	250	70
Typhoid 3T$^+$	900 × 650	950 (periodicity 30)	150	60
Typhoid C4	1000 × 750	1000 (periodicity 30)	200	70
Typhoid 11F	900 × 650	920 (periodicity 30)	150	60
Typhoid 66F	900 × 700	950	150	—
Typhoid phage 2	720 × 660	1100	220	100
Typhoid Vi 1	900 × 950	1100	180	—
Coli T5	750 × 750(650)	1800 (periodicity 44) (1700)	120	30
Typhoid phage 1	750 × 750	1800	90	—
Coli T1	620 × 620(500)	1300(1500)	100	30
Typhoid S1BL	500 × 500	1300	100	40
Pseudomonas Pc	650 × 650	1600 (knob 200)	110	60
Pz	650 × 650	1600 (knob 200)	100	—
Staphylococcus 187	600 × 600	1800 (knob 320)	120	—
Staphylococcus 77	600 × 600	2500 (knob 200)	100	—
Staphylococcus 3B	800 × 600	3000 (knob 200)	100	—
Staphylococcus 6	920 × 400	3000 (knob 200)	100	—
Staphylococcus 71	630 × 630	1700 (knob —)	100	—
Staphylococcus 52	550 × 550	1500 (knob —)	110	—
Streptococcus 3ML	550 × 400	1000	90	—
Coli T3	550 × 550(470)	150(150)	80	—
Brucella	550 × 550	150	—	—
Coli S13	250	—	—	—

* The dimension from the apex to the tail joint is given first, then the width.

† Sheaths were measured in the uncontracted state; where there is no sheath the tail diameter is given.

‡ After Brenner *et al.* (1959).

§ Bracketed figures denote dimensions obtained from shadowed preparations except in the cases of tail periodicity and knob dimensions.

Group 1. *Phages with contractile tail sheaths*

(*a*) *Coli-typhoid phages* 3T$^+$, C4, 11F, 66F (Figs. 1, 2 and Pl. 1, figs. 1–4)

These phages are all morphologically identical except for minor differences in dimensions (Table 1), and are indistinguishable from coli phage T2 (Brenner *et al.* 1959). The chief features are the bipyramidal hexagonal prismatic head, the contractile tail sheath with its cross-striations (spacing 30 Å) shown well in Pl. 1, fig. 3, and the terminal base-plate with several prongs (three are shown in Pl. 1, fig. 2). The central hollow tubular core of the tail is shown in Pl. 1, fig. 4, after contraction of the sheath has occurred. A minor feature is the constriction of the tail at its junction with the head. The appearance of the heads in Pl. 1, figs. 1 and 2, suggest that the DNA is still present, but has been lost in Pl. 1, fig. 4. The head in Pl. 4, fig. 3, also contains the DNA, but the contrast in Pl. 1, figs. 3 and 4, is lower because a rather thin film of potassium phosphomolybdate (this is more transparent than phosphotungstate) was used.

(*b*) *Typhoid phage* 2 (Figs. 3, 4 and Pl. 1, figs. 5–7)

This phage is similar in many ways to the 3-T 2 group, but the morphological differences are distinct. First, the head is much shorter (Table 1) and is of a different shape. The well-preserved empty heads in Pl. 1, figs. 6 and 7, appear to be octahedra, although the possibility that they are icosahedra cannot be ruled out. The tail is the same length as in the 3-T 2 group, and also consists of a core surrounded by a sheath shown contracted in Pl. 1, figs. 6 and 7. No cross-striations can be seen on the uncontracted sheath in this preparation (Pl. 1, fig. 5), although this does not necessarily mean that they are absent, since they are not always visible in coli phage T 2 preparations. The sheaths are readily detachable from the core and three are shown in Pl. 1, fig. 7. One is lying almost end-on and exhibits the cog-wheel appearance associated with the 3-T 2 group sheaths. It thus seems likely that the re-arrangement of the sheath molecules to achieve contraction is similar to that in phage T 2 as suggested by Brenner *et al.* (1959). There is a distinct base-plate attached to the sheath, but unlike coli phage T 2 it possesses no tail pins.

(*c*) *Vi phage* 1 (Figs. 5, 6 and Pl. 1, fig. 8)

This phage has a regular polyhedral head like that of phage 2 but is significantly larger (Table 1). Its tail carries a very conspicuous base-plate.

Group 2. *Phages without contractile tail sheaths*

(*a*) *Coli phage* T 5, *typhoid phage* 1 (Fig. 7, and Pl. 1, fig. 9, and Pl. 2, figs. 10–13)

These two phages are morphologically identical and only one (T 5) is shown. It is the largest in this group (see Table 1) and is calcium-dependent. Here a striking difference was found in appearance, as between particles stained with phosphotungstate and those treated by the experimental uranyl acetate staining described above. No structure is visible on the tail or head in the standard preparation (Pl. 1, fig. 9), but the uranyl acetate preparation (Pl. 2, figs. 10–13) shows up cross-striations on the tail, with a period of 44 Å, rather larger than in the sheath of coli phage T 2 (30 Å). A central core is visible as in the standard preparation. It is not fully understood why the striations appeared in one preparation only, but two explanations are considered possible. The first is that under negative contrast conditions (as with phosphotungstate) the tail subunits are invisible because insufficient contrast is produced. In the case of uranyl acetate, however, it is possible that uranium has been attached to the protein subunits and a true stain obtained in addition to negative contrast; thus the subunits would show in one case and not the other. The second possible explanation is that the subunits are partially disorganized by the change in pH values which the particle has suffered, so that they are rendered visible by a structural change. In either case there is no doubt that the molecular organization of the tail is shown. A disorganized tail (Pl. 2, fig. 10, arrowed) suggests that the subunits are of tubular form.

Subunits are also visible in the head in the uranyl acetate preparation shown in Pl. 2, figs. 10–13. It is clear from these electron micrographs that the molecular structure of the head has undergone considerable disorganization, due no doubt to the low pH value. It seems that it is only this disorganization which enables the subunits to be seen, since in a fully organized state they are probably too closely packed. They appear to be broadly similar in appearance to those found on the surface of Herpes virus by Horne, Brenner, Waterson & Wildy (1959), though very much smaller. They consist of hollow tubes (similar to the tail subunits) about 100 Å long and 50 Å in diameter. It is not possible to state the method of packing as the degree of disorganization is too great, though some regularity is visible in Pl. 2, fig. 11. Most of the subunits in Pl. 2, fig. 12, are visible end-on, and those in Pl. 2, fig. 13, are lying on their sides. It is clear that T5 would repay further more detailed investigation.

(*b*) *Coli phage* T1 *and typhoid phage* S1BL (Fig. 7, and Pl. 3, figs. 14–17)

These two types are morphologically identical save for a slight difference in the size of the head (Table 1) and in the absence of the thin neck between the head and the tail in 71. Plate 3, figs. 14 and 15, show that the tail of phage S1BL is built up of an inner hollow tube, surrounded by a sheath which shows no signs of contraction even when the DNA has been released as in Pl. 3, fig. 14. At its junction with the head a small knob is visible. A well-marked pattern is visible in the deflated head of S1BL in Pl. 3, fig. 14. This pattern is visible in several other particles which have lost their DNA (Pl. 3, figs. 17 and 19–21).

(*c*) *Pseudomonas phages* Pc *and* Pz, *Staphylococcus phages* 187, 77, 3B, 6 (Figs. 8, 9, 12, and Pl. 3, figs. 18–23, and Pl. 4, figs. 24–30)

These can be subdivided into phages with very long tails (6, 3B, 77) and those with shorter tails (187, Pc, Pz). Of the latter Pc is identical with Pz. Phages 187 and T1 resemble these two, apart from the terminal knob. The heads are regular polyhedra and the tails are hollow tubes. The lumen of the tail can be seen when the DNA has been discharged (Pl. 3, fig. 20), but cannot be seen when the DNA is present (Pl. 3, fig. 18).

The tail knobs on phages 187 and 71 are much larger than those of Pc and in Pl. 3, fig. 23 (phage 187) the knobs have a six-sided appearance.

Phage 77 (Pl. 3, fig. 19) has an unusually long tail and when the DNA has been discharged can be seen to be hollow throughout its length; it is attached to a rather small polygonal head. Phages 3B and 6 (Pl. 4, figs. 26–30) are especially remarkable for the length of the tail, 3000 Å is the largest yet found, and the head of a length equal to twice its width. Satisfactory micrographs of the head of phage 3B have not been obtained but from examination of the shape of the empty head it is tentatively concluded that the intact particles resemble the drawing in Fig. 12. The terminal tail knobs are small in comparison to those of phage 187.

(*d*) *Streptococcus phage* 3ML (Fig. 11, and Pl. 4, figs. 24, 25)

This is a small phage of a shape not found previously. Its head shows no sharp angularity and its tail is devoid of sheath or neck. The tail in the DNA-less phage (Pl. 4, fig. 24) can be seen to be hollow.

Group 3. *Phages with very short tails, coli phage* T 3 (Fig. 10, and Pl. 4, figs. 31–33) *and Brucella phage* (Pl. 4, fig. 34)

Both these phages have polygonal heads, six-sided in plan view, 650 Å across. They possess very short wedge-shaped tails, 140 Å long, attached at one corner of the head. It is particularly interesting that the only Brucella phage available (Brinley Morgan *et al.* 1960) should be the only example of this morphological type to be described outside the coli-typhoid group.

DISCUSSION

The results shown here are primarily of morphological interest, but a detailed knowledge of phage morphology is essential before any attempt can be made to elucidate the mechanism of injection. It is obvious from the foregoing that phages which lack contractile sheaths must use some other means of penetrating the bacterial cell wall; this is at present obscure. A study of Table 1 (Dimensions) is of taxonomic interest. The present state of phage taxonomy is extremely confused, and it can be seen that neither pure morphology nor differences in host species can provide a complete answer as a method of classification. For example, if morphology were used, the group of phages resembling coli phage T 2 would all be labelled as one species or type, but in fact they require different host cells. Morphology may well be a valuable aid of classification, however, and it can be seen that the different types studied here fall into fairly well-defined groups.

There are many questions to which the negative contrast method does not provide an answer, such as the shape of the head. It unfortunately only shows this in two dimensions and though a hexagonal outline is revealed it is not possible to state the geometric form which the head takes without a very high quality shadowed preparation using shadowing from two directions (Smith & Williams, 1958). The only other way to elucidate the true head shape is to study the packing of subunits, but these are not visible in an organized state. There is no doubt that the negative contrast method can be varied considerably as suggested by Nixon (1960) and different embedding compounds used. This has been amply confirmed by the difference in appearance between the phosphotungstate and uranyl acetate preparations of T 5. The treatment of phage particles at different pH values and the subsequent study of their molecular structure is clearly the next step in the application of the method. There is little doubt that the technique is an extremely valuable aid in phage research and should enable a great deal of fundamental knowledge to be obtained about this form of virus.

With the exception of the S 13 phage (Hall, Maclean & Tessman, 1959), of which the 'tail' if any is as yet undisclosed, all phages have in common a 'head' and a 'tail', the sizes and shapes of which can vary considerably. There seems no reason to doubt that the head contains the DNA of the phage and that the tail, at least in cases where there is a knob or base-plate, carries at its distal end the device which enables the phage to adsorb specifically to its host cell.

Phage tail structures vary enormously in their complexity from the elaborate contractile sheath, base-plate and fibres of Group 1 to the apparent simplicity of Group 3. This simplicity may be only apparent and if higher resolution could be obtained a complexity at molecular level equal to Group 1 might be found. However, apart from Group 3 in which detail is lacking, all phage tails are basically tubes, the length of which may differ from strain to strain. The tube is not visibly hollow unless the head has lost its DNA which implies that the head DNA, when it departs under the condition of the preparation technique, is completely ejected from both head and tail. This is seen in Pl. 1, figs. 4, 6, 9, and Pl. 2, figs. 10, 14, and others. The only information available about the fine structure of this tube is that given in Pl. 2, fig. 10, showing T 5 treated with uranyl acetate. A banded structure with a periodicity of 44 Å is visible. In some phages the tail tube is surrounded with either a contractile (Group 1) or a non-contractile sheath (Group 2, some members). The non-contractile sheath is visible in Pl. 3, figs. 14 and 22, where it does not cover the first part of the tube as it emerges from the head. Certain phages, notably T 1 (Pl. 3, fig. 17), T 5 (Pl. 2, fig. 10) and 3 ML (Pl. 4, fig. 25), show no sign of comparable thickening of, or sheath around, the tube.

The contractile sheath of phage C 4 (Pl. 1, figs. 3, 4) is similar to that of T 2 (fully described by Brenner *et al.* 1959), especially in showing a periodicity of 30 Å. Brenner *et al.* believed the T 2 sheath to be a helix; if so, the C 4 sheath is probably also a helix.

Tails may be terminated in three ways. The end-plate with prongs, as described by Brenner *et al.* (1959), for phage T 2, also well shown in 11 F (Pl. 1, figs. 1, 2) and very markedly in Vi 1 (Pl. 1, fig. 8), is the most complex terminal device. In Pl. 1, figs. 6–8 the end-plate is seen still attached to the contractile sheath even after contraction has taken place.

The second device for the termination of phage tails is the knob apparent in Pl. 3, figs. 18–22, and Pl. 4, figs. 28 and 29. This knob appears to be polygonal, it is hexagonal in plan view when derived from phage 187 (Pl. 3, fig. 23). In this case the knobs were sometimes found attached to linear structures about equal to the phage tail in length but of reduced diameter. These structures were first observed in staphylococcus phage K by Hotchin (1954) who believed them to be plugs which fitted into the hollow tail of the phage. The present studies have not yet been able to confirm or deny this suggestion.

Finally the tails may terminate in what may be a rounded or pointed tip (Pl. 2, fig. 10, and Pl. 3, fig. 17). Plate 3, fig. 16, shows a tail tip of this sort turned upwards and viewed end-on.

The heads of the phages in all cases, except 3 ML (Pl. 4, figs. 24, 25) show or have shown in other pictures a hexagonal appearance in plan view. Most are regular hexagons but a few are slightly elongated (Group 1) and some (Pl. 4, figs. 27–29) show considerable elongation. The angularity frequently survives the loss of the DNA, as shown in Pl. 1, figs. 6, 7, Pl. 2, fig. 13, and Pl. 3, figs. 21 and 22, indicating a degree of rigidity in the head membrane and suggesting that the angular shape of the phage head is due to the arrangement of the subunits from which it is built, rather than to the packing of the DNA which it surrounds.

The fine structure of the head membrane is never visible in the phosphotungstate-treated specimens, but in the case of the uranyl acetate-treated phage T 5 (Pl. 2, figs. 10–13) the size and shape of the subunits forming the membrane can just be discerned. They appear to be short, hollow cylinders about 100 Å long and 50 Å in diameter and are disorganized, presumably due to the staining procedure.

A membraneous structure is visible within the main structural envelope of the head of some phages, particularly in Pl. 3, figs. 14, 16, 17 and 19–21, where the DNA has been discharged. It suggests a collapsible elastic membrane normally surrounding the DNA just inside the rigid structural membrane of the head and as this is always seen attached to the origin of the tail tube there is the possibility that this membrane, in tension, provides the driving force to expel the DNA from the phage particle during infection of a cell.

The authors wish to thank Mr R. W. Horne for valuable suggestions. One of them (D. E. B.) is grateful to Dr T. E. Allibone, F.R.S., Director of the Research Laboratory, Associated Electrical Industries, for permission to publish this paper.

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EXPLANATION OF PLATES

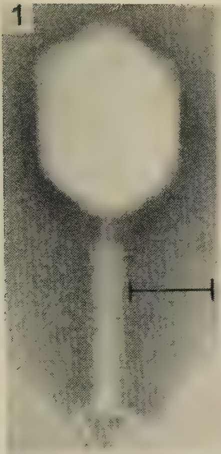
Note. 'Active' indicates that DNA is present in the head and 'inactive' denotes that it is not and that the particle is presumably non-infective. 'PTA' indicates the phosphotungstate method and 'PMA' the phosphomolybdate method. Scale marks represent 500 Å.

PLATE 1

Figs. 1, 2. Typhoid phage 11F, active, PTA method, × 250,000.
Fig. 3. Typhoid phage C4, active, PMA method, × 250,000.
Fig. 4. Typhoid phage C4, inactive, PMA method, × 250,000.
Fig. 5. Typhoid phage 2, active, PTA method, × 250,000.
Figs. 6, 7. Typhoid phage 2, inactive, PTA method, × 250,000.
Fig. 8. Typhoid phage Vi1, active and inactive, PTA method, × 145,000.
Fig. 9. Coli phage T5, inactive, PTA method, × 250,000.

PLATE 2

Fig. 10. Coli phage T5, inactive, uranyl acetate method, × 250,000.
Figs. 11–13. Coli phage T5, inactive, uranyl acetate method, × 500,000.



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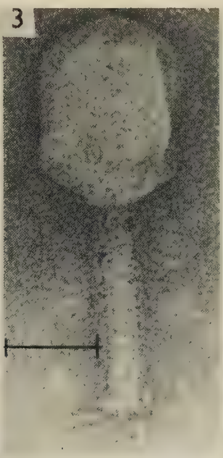

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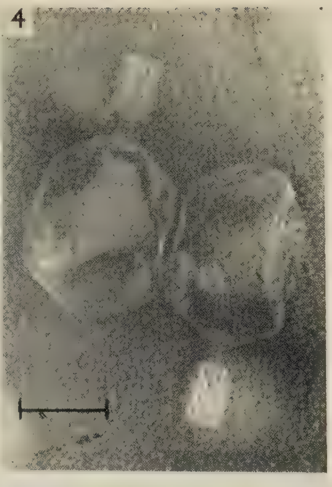

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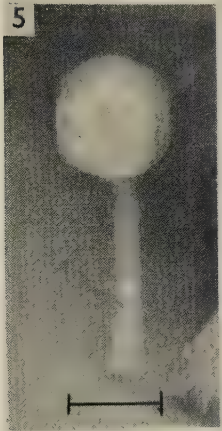

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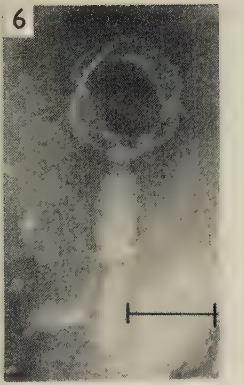

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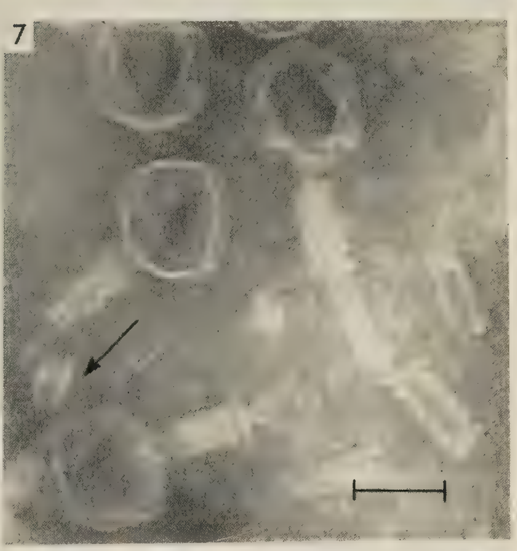

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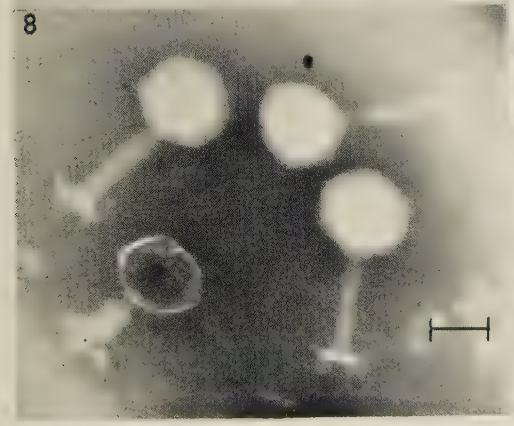

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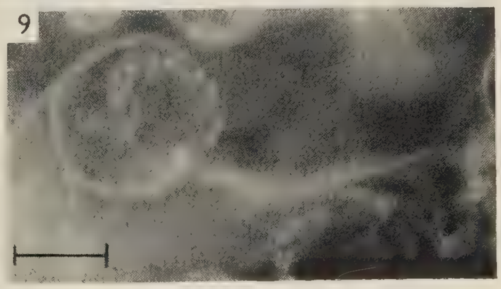

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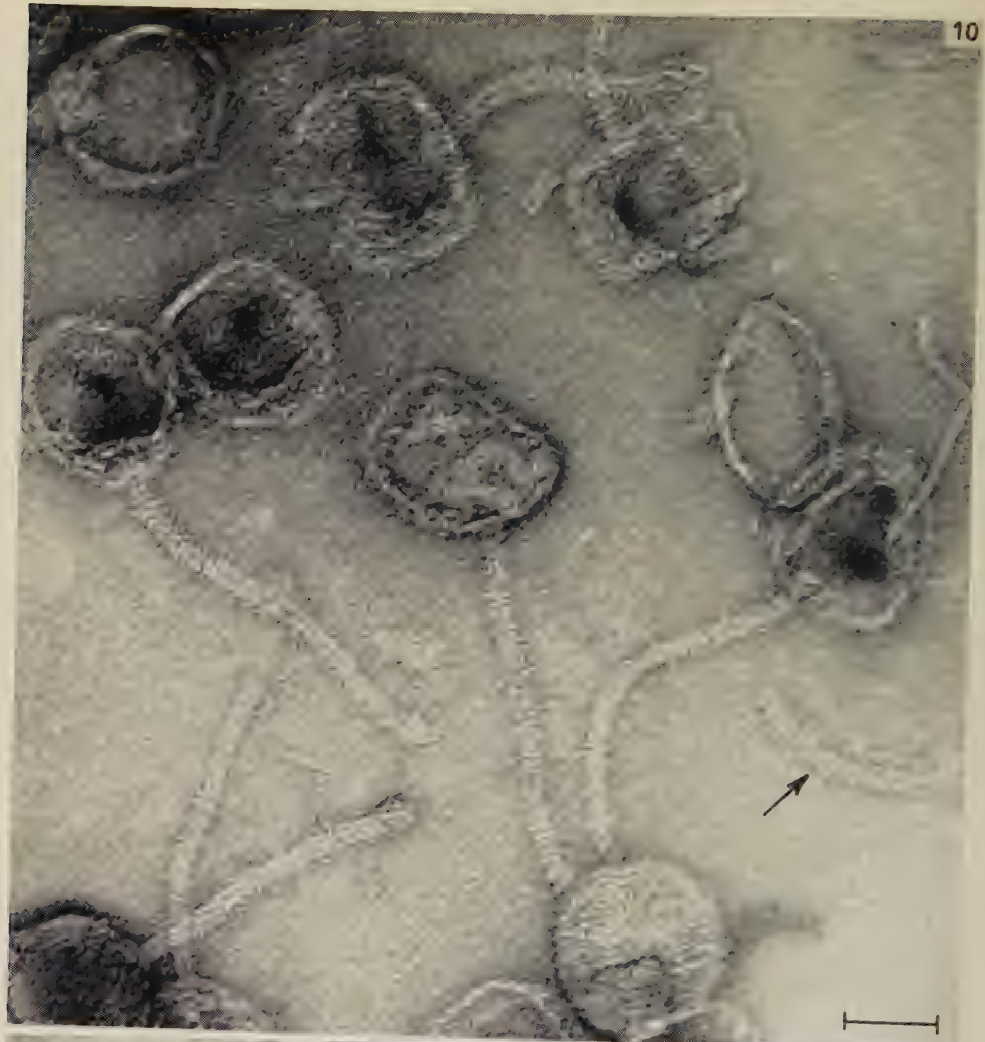

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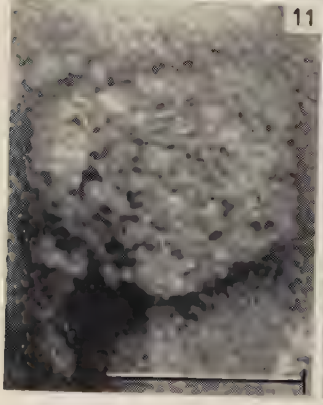

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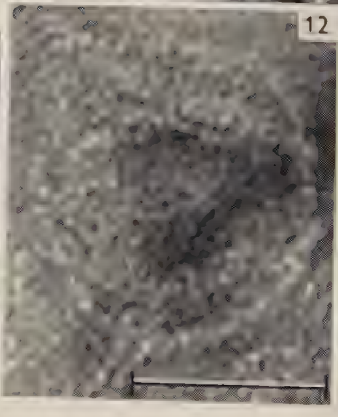

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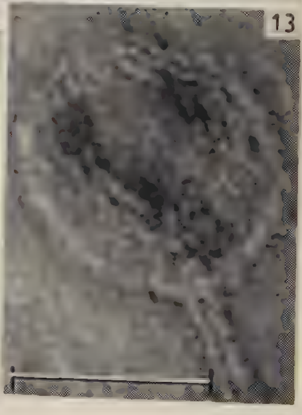

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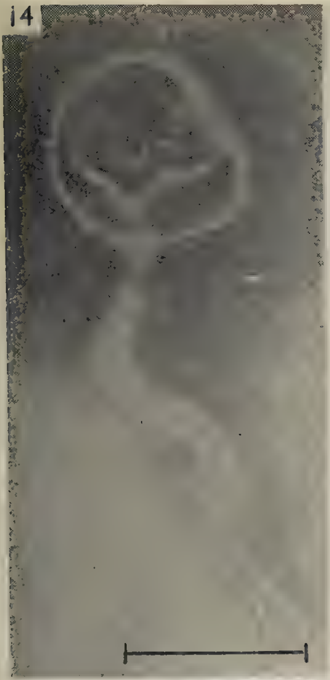

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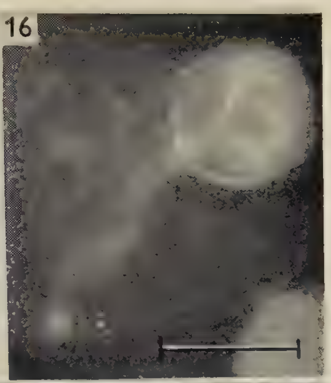

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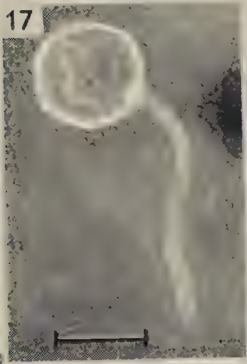

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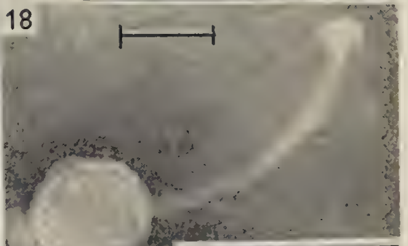

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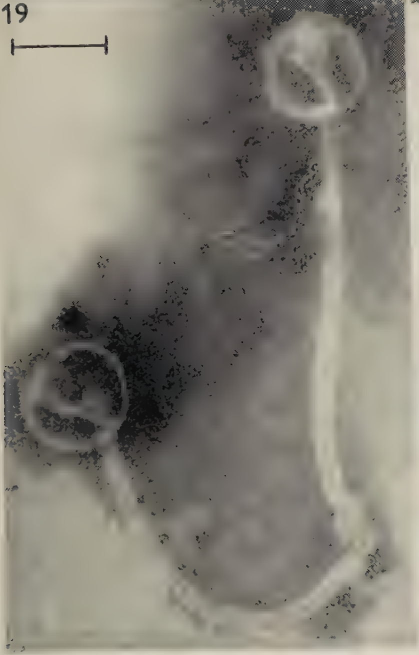

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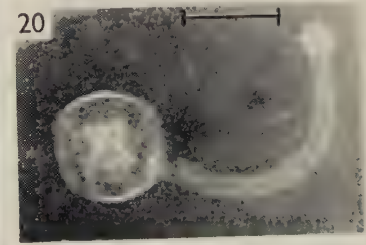

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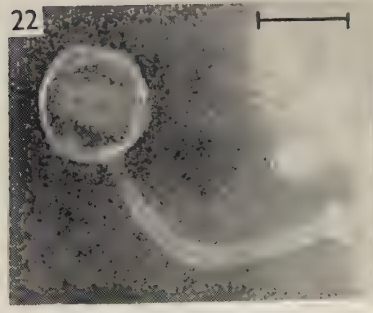

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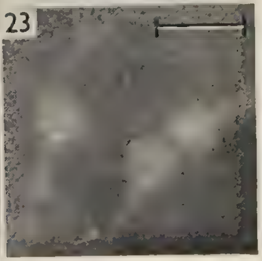

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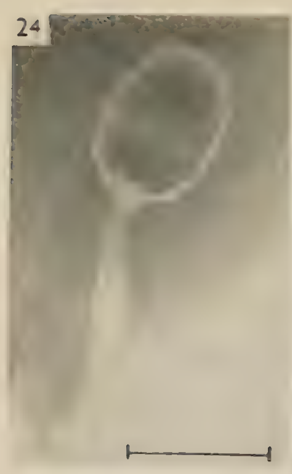

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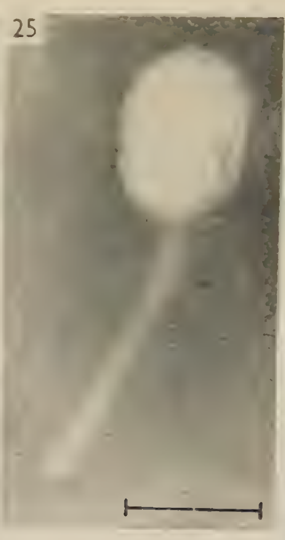

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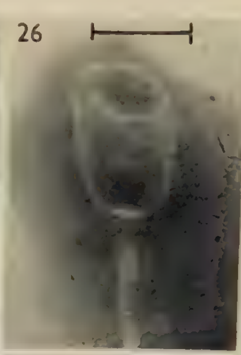

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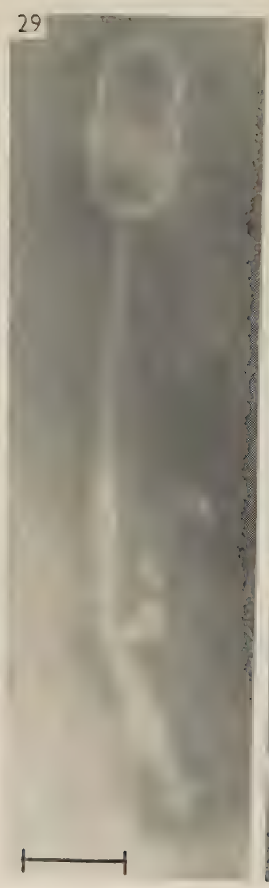

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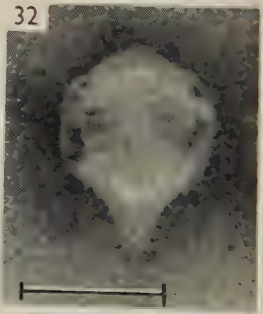

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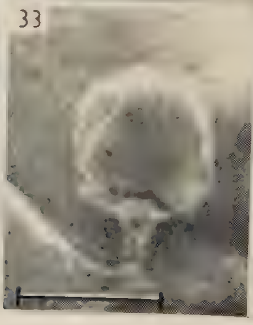

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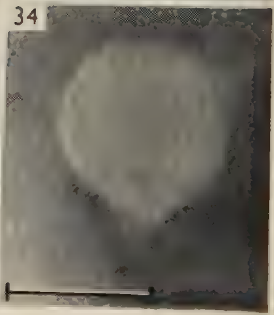

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PLATE 3

Fig. 14. Typhoid phage S1BL, inactive, PMA method, × 500,000.
Fig. 15. Typhoid phage S1BL, active, PMA method, × 250,000.
Fig. 16. Coli phage T1, inactive, PTA method, × 375,000.
Fig. 17. Coli phage T1, active, PTA method, × 250,000.
Fig. 18. Pseudomonas phage Pc, active, PTA method, × 250,000.
Fig. 19. Staphylococcus phage 77, inactive, PTA method, × 250,000.
Fig. 20. Pseudomonas phage Pc, inactive, PTA method, × 250,000.
Fig. 21. Staphylococcus phage 187, inactive, PTA method, × 250,000.
Fig. 22. Pseudomonas phage Pc, inactive, PTA method, × 250,000.
Fig. 23. Staphylococcus phage 187, tail knobs, PTA method, × 250,000.

PLATE 4

Fig. 24. Streptococcus phage 3ML, inactive, PTA method, × 375,000.
Fig. 25. Streptococcus phage 3ML, active, PTA method, × 375,000.
Figs. 26, 27. Staphylococcus phage 3B, inactive, PTA method, × 250,000.
Fig. 28. Staphylococcus phage 6, active, PTA method, × 250,000.
Figs. 29, 30. Staphylococcus phage 6, inactive, PTA method, × 250,000.
Figs. 31 and 32. Coli phage T3, active, PTA method, × 375,000.
Fig. 33. Coli phage T3, inactive, PTA method, × 375,000.
Fig. 34. Brucella phage, active, PTA method, × 375,000.

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An Improved Method for Viable Counts of Bacteria of the Ovine Rumen which ferment Carbohydrates

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SUMMARY

A method is described which is suitable for routine viable counts of rumen bacteria capable of fermenting various carbohydrates. The contribution of different steps in the preparation of the medium towards the final redox potential was determined and the choice of a suitable reducing agent, redox indicator and source of rumen fluid supplement is discussed. In a sheep on a constant practical ration, the variation in numbers of cellulolytic bacteria was greater than the differences between five successive samples withdrawn at one time, and much greater than the variation due to the method.

INTRODUCTION

There has been considerable interest in recent years in the role played by the bacterial population of the rumen in the digestive processes of the host animal. These rumen bacteria may be divided into three groups: (1) functional bacteria; (2) bacteria accidentally introduced with food and water; (3) potentially harmful bacteria normally present in insignificant numbers (Gilchrist & Clark, 1957). In studies on the influence of the ruminal flora on the metabolism of the ruminant, the main interest lies with the first-mentioned group, and several authors have proposed sets of criteria for establishing whether a bacterial strain is a functional member of the rumen population or not (Gall & Huhtanen, 1951; Briggs, 1955; Bryant, 1959). These criteria are all elaborations on the two fundamental principles laid down by Elsden & Phillipson (1948), namely, that a functional ruminal organism is one which performs a chemical reaction known to occur in the rumen and is present in numbers sufficient to perform this reaction at a rate comparable with that occurring in the rumen. One of the first requirements in the study of functional rumen bacteria is thus the ability to determine the number of bacteria capable of bringing about a certain biochemical reaction.

Although the importance of this type of determination was recognized more than a decade ago, very few quantitative studies have been made. One of the reasons for this is that there is no satisfactory routine method for performing counts of this nature. It is now generally recognized that many functional rumen bacteria are closely adapted to their peculiar environment and cannot be cultivated by conventional bacteriological methods. To obtain valid viable counts of the different metabolic groups of rumen bacteria, it is, therefore, essential to provide cultural conditions which closely resemble those existing in the rumen of the experimental animal. The most important conditions are: (1) the average temperature of the rumen (*c.* 38·5°) as the temperature of incubation; (2) an inorganic salt composition of the medium similar to that of the ruminant's saliva; (3) bicarbonate + carbon dioxide as the main buffer system of the medium; (4) a certain percentage of rumen fluid as a source of growth factors and reducing agents; (5) the maintenance of a low redox potential in the medium. The importance of some of these factors in the cultivation of functional rumen bacteria was discussed in detail by Bryant (1959).

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Bryant & Burkey (1953*a*, *b*) described a method which took into account the above-mentioned cultural requirements and which enabled them to determine the number of bacteria capable of fermenting a variety of carbohydrate substrates. However, the method involves the quantitative isolation of the colonies obtained by inoculation of high dilutions of rumen contents on a non-selective solid medium and subsequent determination of the fermentation reactions of the resultant cultures and is therefore much too cumbersome for routine determinations of the composition of the bacterial population of the rumen under different dietary conditions. In view of this, it was decided to follow a more direct method by counting the number of colonies which developed on selective solid media containing single carbohydrate substrates. A certain amount of overlapping of the counts on the different media was to be expected, but this would not be too serious as the results were not intended for estimation of total counts. The medium and techniques of Hungate (1950) were selected as being the most suitable as the basis of the proposed method. However, these were intended for isolation studies and were not convenient for routine counts. They were therefore modified to give greater speed and accuracy. At the same time, more rigorous anaerobic precautions and a more sensitive redox indicator were introduced to ensure consistent growth of some of the predominant types of cellulolytic bacteria. The present communication describes the method finally evolved as well as the rationale of the various steps in the procedure and the reproducibility and limitations of the method.

METHODS

Determination of oxidation-reduction potentials in media

The potentials were measured with a Metrohm 280-E battery-operated pH and millivolt meter which has an accuracy of ± 10 mV. The electrode system was: unknown solution Pt/KCl bridge/Ag/AgCl, saturated KCl. The platinum and reference electrodes were combined in a single probe provided with a standard ground-glass joint. The conventional method of cleaning platinum electrodes by immersion in hot strong nitric acid followed by rinsing with distilled water resulted in a very slow equilibration of the electrode with strongly reducing systems. This situation was remedied by subjecting the platinum surface to an electrolytic treatment immediately before use. The electrode was immersed in 100 ml. N-HCl solution containing 1 drop of Teepol and connected to the negative pole of a 6-V. dry battery. A graphite rod served as anode and the electrolysis was continued for 3 min., after which the electrode was rinsed with distilled water and immediately fitted into the lid of a Metrohm 2-part titration vessel which had previously been purged with oxygen-free CO_2. The medium of unknown E_h value was introduced into the titration vessel under strictly anaerobic precautions and was stirred by means of a current of oxygen-free CO_2. The vessel was also fitted with a combined glass and reference electrode so that E_h and pH values could be recorded simultaneously.

Method for making viable counts

The methods finally adopted were as follows:

Gassing. Air was displaced from solutions, media and culture vessels by gassing with sterile oxygen-free CO_2 or, where the high solubility of this gas in aqueous solutions was objectionable, with N_2. The commercial gases were freed from O_2 by passing them over palladinized copper turnings held at 300°. With the aid of a manifold and flowmeters, six equal currents of oxygen-free gas could be obtained, which were rendered germ-free by passing through sterilized filters. These consisted of the barrels of 2 ml. Luer Lock syringes stuffed with cotton wool. For gassing the smaller vessels, these filters were provided with 19-gauge hypodermic needles of sufficient length to reach the bottoms of the vessels. Rubber-stoppered bottles were gassed by inserting the needle between the rubber stopper and the neck of the bottle. They were sealed without entry of air by carefully withdrawing the needle with the right hand while holding down the stopper with the left thumb and forefinger. The rate of gassing was usually approximately 12 l./hr.

Substrates. The final concentrations of the substrates employed in this study were: soluble starch (Merck's 'pro analysi') 1·2 % (w/v); glucose (Merck's 'for bacteriology') 1·0 % (w/v); d(+) xylose (Merck's 'pure chemicals') 1·0 % (w/v); a finely ground suspension of acid-treated cellulose prepared according to Hungate (1950) 1·2 % (w/v).

Rumen fluid supplement. Rumen ingesta were withdrawn by suction tube from a fistulated sheep before morning feeding. As a rule the rumen fluid was obtained from animals on a lucerne hay diet (see below). The ingesta were strained through a double thickness of cheesecloth and the fluid collected in a 1-pint plastic bottle which was completely filled. The bottle was sealed with a rubber bung and chilled in crushed ice for at least 30 min. to bring gaseous fermentation to a standstill. In order to keep the coarser particles of the rumen contents out of the medium where they might later be confused with small colonies, the bottle was centrifuged at 1200 *g* for 20 min. When required, the supernatant fluid was carefully removed by means of a 50 ml. syringe with a 4 in. cannula while the gas space above the fluid was purged with oxygen-free CO_2 to prevent absorption of atmospheric O_2.

The composition of the medium was as follows: $(NH_4)_2SO_4$, 5 % (w/v) stock solution, 5 ml.; K_2HPO_4, 5 % (w/v), 5 ml.; KH_2PO_4, 2 % (w/v), 5 ml.; $CaCl_2$, 0·5 % (w/v), 5 ml.; $MgSO_4.7H_2O$, 0·5 % (w/v), 5 ml.; NaCl, 10 % (w/v), 5 ml.; Antifoam B (Dow Corning), 10 % (v/v) emulsion in distilled water, 5 ml.; indigodisulphonate, 0·05 % stock solution, 5 ml.; cysteine HCl, 250 mg.; $NaHCO_3$, 2.6 g.; processed rumen fluid, 150 ml.; solution or suspension of the substrate to give the final concentration stated above; deionized water to 500 ml.

Cellulose and starch were sterilized together with the basal medium; glucose and xylose were added to the molten agar after sterilization in the form of 25 % (w/v) Seitz-filtered stock solutions.

Preparation of medium. Agar (7·5 g.) was weighed into a 40 oz. screw-capped bottle, the appropriate volume of distilled water was added and the bottle heated in a boiling water-bath until the agar was dissolved. When cellulose or starch was to be substrate, 150 ml. of a hot 4% (w/v) suspension or solution were added to the molten agar at this stage, followed by the above-mentioned quantities of inorganic salt solutions, redox indicator and anti-foaming agent. Oxygen-free CO_2 was then bubbled through the hot mixture by means of a 16-gauge stainless-steel cannula inserted between the neck of the bottle and a well-fitting rubber stopper. The rate of bubbling was fast enough to produce turbulence in the medium.

After gassing for a minimum of 5 min., the bottle was removed from the water-bath and cysteine.HCl added to the medium, followed by 150 ml. of centrifuged rumen fluid. The medium was now cooled to *c.* 47° before the $NaHCO_3$ was added. After replacing the rubber stopper, gassing was continued for another 10 min. to saturate the medium with CO_2. The cannula was then carefully withdrawn to prevent re-entry of air, and the rubber stopper sealed down by a screw cap with paper liner. The medium was immediately sterilized at 120° for 25 min. and then allowed to cool to 47° before distribution.

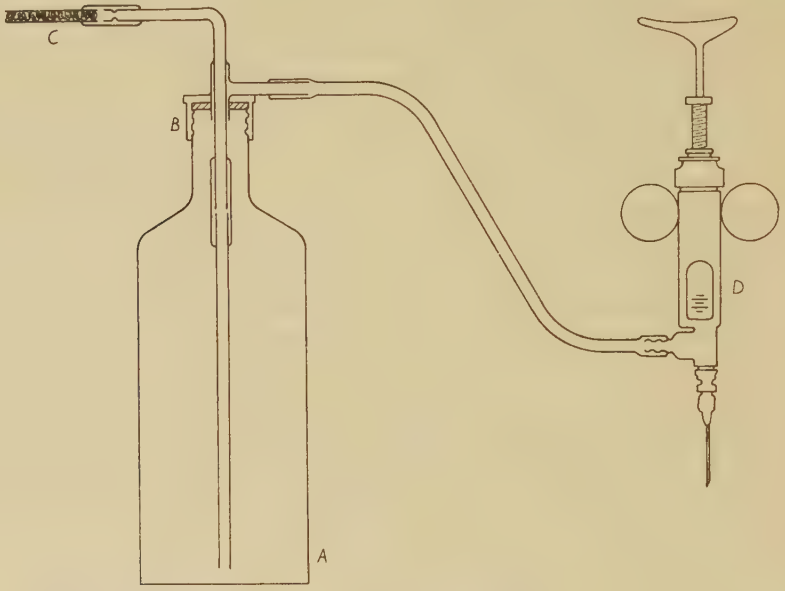

Fig. 1. Automatic pipetting outfit. *A*, 40 oz. McCartney bottle (United Glass Bottle Manufacturers, London); *B*, filterhead (Gallenkamp, London) with inlet tube extended to the bottom of the bottle by means of glass tubing; *C*, cotton-wool filter; *D*, automatic syringe, 5 ml. capacity (Milborrow and Co., Pietermaritzburg), connected to *B* by means of latex tubing.

Where glucose or xylose was to be the substrate, 20 ml. of a 25% (w/v) Seitz-filtered stock solution were injected into the bottle, after sterilization, through the rubber stopper, and thoroughly mixed with the medium by shaking.

Distribution of medium over dilution bottles. Moist 1 oz. screw-capped bottles were provided with well-fitting rubber stoppers and flushed out with oxygen-free N_2 for 5 min. as described under 'Gassing' above. The screw caps were then fitted over the rubber stoppers and the bottles sterilized at 120° for 30 min., together with the automatic pipetting outfit shown in Fig. 1. After cooling to room temperature, the pipetting outfit was connected to the gas manifold and, with CO_2 passing through the inlet tube, the empty bottle was quickly replaced by a bottle of sterile molten agar medium at 47°, with aseptic precautions. The filter head was left slightly loose to allow gas to escape between the rim of the bottle and the filter head, and the bottle was purged with oxygen-free CO_2 for another 5 min. After tightening the filter head, air was displaced from the rubber tubing and the syringe by depressing and releasing the plunger of the pipetting outfit 20 times. By inverting the bottle and working the plunger, the medium was alternately fed into the syringe and injected into the dilution bottles in 9·0 ml. quantities through the rubber stoppers. The bottles were stored at room temperature. When required, the medium was melted and the solid screw caps were replaced by sterile caps with two $\frac{1}{16}$ in. perforations *c.* $\frac{1}{2}$ in. apart.

Modified cellulose medium. Sodium dithionite was substituted for cysteine in the cellulose agar to give a final concentration of 0·003 % (w/v). As dithionite is decomposed by heat, 0·2 ml. of a freshly prepared 0·15 % solution in oxygen-free water under N_2 pressure was added to 8·8 ml. molten agar in the dilution bottles shortly before use. The solution could be conveniently sterilized by passing it through a Swinny filter adapter (Becton, Dickinson and Co., Rutherford, N.J.) containing a $\frac{1}{2}$ in. Seitz filter mat.

Sample dilutions. The samples of rumen ingesta were taken by means of a suction tube from fistulated animals. The ingesta were strained through a double thickness of cheesecloth and the liquor collected in a glass-stoppered bottle which was completely filled and immediately returned to the laboratory. By means of a Luer Lock type syringe with a 20-gauge × $1\frac{1}{2}$ in. needle, 1 ml. of sample was injected into a dilution bottle containing 9 ml. of molten agar medium at 47°; thorough mixing was obtained by rolling the bottle between the hands and simultaneously turning the wrists through an angle of 180°. With a fresh sterile syringe 1 ml. of the first dilution was then withdrawn through a second perforation of the rubber stopper —to avoid contamination of the needle with a trace of the original sample—and injected into the next bottle. In this way a series of tenfold dilutions was prepared, the degree of dilution required to obtain a countable number of colonies in the roll bottles depending on the substrate used and the composition of the ruminal flora of the particular animal.

Beginning with the highest dilution, 4 ml. of each of the last four dilutions was drawn into a Luer Lock syringe and dispensed over four sterile $\frac{1}{4}$ oz. rubber-stoppered roll bottles which had previously been filled with CO_2. In order to remove any O_2 which might have been introduced during this process, the bottles were immediately purged with oxygen-free CO_2 for 2 min., while partially immersed in the 47° water-bath. The bottles were then spun, four at a time, in an indirectly water-cooled solidification apparatus (Apparatenfabriek van Dooren, de Bilt, Netherlands) for 3 min. to obtain a uniform film of agar of *c.* 2 mm. thickness along the walls of the roll bottle. The bottles were incubated upside-down to allow the water of syneresis to drain off.

Incubation times at 38·5°. Starch medium 16 hr.; glucose and xylose media 20 hr.; cellulose medium 5–42 days depending on the rate of growth of the cellulolytic bacteria in the sample.

Counting. The counting device of Apparatenfabriek van Dooren was used; this permits scanning of the surface of the bottle in spiral fashion. The bottles were illuminated from below and the colonies were viewed at a magnification of ×10. Whenever the redox indicator showed that a bottle had absorbed O_2, the colony count was disregarded in calculating the average number for that particular dilution. On cellulose media only those colonies which were surrounded by a visible zone of clearing were counted. Similarly, the colony counts on starch media were made specific by spreading a few drops of Lugol's iodine solution over the agar and counting only those colonies which were surrounded by a concentric colourless zone.

RESULTS AND DISCUSSION

Redox potential of the medium

Although it is now generally recognized that the maintenance of a low redox potential in the medium is of utmost importance in the cultivation of predominant rumen bacteria, few data have been published on the actual E_h values obtained in the media by the use of different reducing agents, as compared with the potentials prevailing in the rumen of the experimental animal from which the samples were to be taken for cultivation. Huhtanen, Rogers & Gall (1952) used cysteine as reducing agent in their sample-diluting fluid and in their medium. In the former, they obtained a redox potential of -200 mV. at pH 6·8 which is well above the E_h value which they reported for the rumen contents of cattle on a high roughage ration, namely, -300 mV. (Huhtanen & Gall, 1953). Nevertheless, these authors succeeded in obtaining growth of several different types of rumen bacteria from high dilutions of the sample. On the other hand, Smith & Hungate (1958) found that they could not cultivate the methane bacteria from the rumen of cattle unless the redox potential of the medium was low enough to reduce benzylviologen (E_0' -359 mV. at pH 7·0) which was included as indicator. The E_h value of the rumen contents which served as the inoculum for their isolations varied between -335 and -346 mV.

In the early stages of the present investigation Hungate's (1950) basal medium containing 0·01 % (w/v) cysteine hydrochloride was used. On this medium consistent counts were obtained of the groups of bacteria which fermented starch, glucose and xylose and also of the rod-shaped cellulolytic bacteria predominating in rumen contents of sheep on teff hay. When this type of medium was inoculated with rumen fluid from sheep fed lucerne hay, in which cocci appear to be the predominant cellulolytic organisms, rapid growth and complete digestion of the cellulose was usually observed in the first dilution. However, instead of the expected tenfold decrease in the number of colonies from one dilution to the next, there was an abrupt break at the second or third dilution and no growth whatsoever beyond that. The possibility existed that this phenomenon was due to a heat-labile growth factor normally present in rumen fluid which was destroyed by heat sterilization of the medium, then reintroduced with the inoculum only to be diluted out with progressive tenfold dilutions. However, this explanation was ruled out by the fact that good growth of the same bacteria was obtained even in the high dilutions when cellobiose was substituted for cellulose. This observation suggested another possibility, namely, that the initiation of the growth of the cocci on media containing a refractory substrate such as cellulose required lower redox potential values than on readily available carbohydrates such as cellobiose. As a first step in testing this hypothesis, the contribution of the different steps in the preparation of the Hungate type medium to the final E_h value was examined. Table 1 shows a typical set of measurements.

From these measurements it appears that the redox potential of the medium was determined by the rumen fluid supplement and that the contribution of the cysteine would be an improved poising capacity rather than a lower E_h value. It may also be seen that the E_h of the medium rose during the sterilization process to approximately that obtained by the addition of cysteine, with the result that the final potential was considerably higher than that of the rumen contents of the animal from which the sample was taken.

In the course of these measurements it was discovered that the method of removing oxygen from commercial gases by passing these through wash bottles containing a chromous acid solution, as recommended by Hungate (1950), was not effective in the case of our bottled carbon dioxide. When this gas was used to stir the medium in the titration vessel during E_h measurements, the redox potential became progressively more positive, while this tendency ceased as soon as the gas flow was interrupted. A furnace containing palladinized copper turnings at 300° was therefore substituted for the chromous acid absorption train and, when the effluent gas from this set-up was passed through a weakly poised medium of low redox potential, no change in potential occurred within 15 min., thus indicating that the residual oxygen content was very low.

Table 1. *The contribution of different steps in the preparation of the Hungate-type medium to the final* E_h

Step	pH value	E_h (mV.)
(1) 70 ml. boiled out basal medium in equilibrium with oxygen-free CO_2	6·8	+55
(2) 10 mg. cysteine.HCl added	6·8	−210
(3) Clarified rumen fluid supplement from a sheep fed lucerne hay	—	−300
(4) 30 ml. clarified rumen fluid added to basal medium	6·8	−271
(5) Medium after sterilization at 120° for 20 min.	6·7	−160

Choice of reducing agent. Inspection of the E_0' values of such biological reducing agents as glutathione, ascorbic acid and thioglycollate indicated that they could not be expected to be superior to cysteine in the production of low redox potentials in our media. However, the novel biological method used by Smith & Hungate (1958) to obtain good poising capacities at low E_h values by growing *Escherichia coli* in the medium and subsequently killing this organism by a pasteurization process without loss of reducing power, looked promising. Unfortunately, this method did not work at our hands as the strain of *E. coli* used would not grow in our medium. However, another method suggested by Smith & Hungate was applied with better success, namely, the use of 0·003 % (w/v) sodium dithionite. It was pointed out by these authors that the low concentration of dithionite tolerated by their rumen methane bacteria resulted in a poorly poised medium which tended to give erratic growth. Nevertheless, we found that the low potentials imparted by dithionite to the medium (−400 to −440 mV. at pH 6·8) could be maintained throughout the procedures of our techniques provided that sufficiently strict anaerobic precautions were taken. Owing to its heat lability, the dithionite had to be added in the form of a freshly prepared Seitz-filtered solution to the molten agar medium immediately before inoculation.

On the dithionite-containing media, good counts of the cellulolytic cocci from sheep fed on lucerne hay were obtained. It was found that these cocci would also grow on cellulose media without added dithionite, provided that the carbon dioxide used for gassing the medium and the cultures had a very low oxygen content. However, the growth of these organisms was more consistent in the presence of dithionite. The use of the modified cellulose medium containing dithionite was, therefore, adopted as a standard procedure for counting all types of cellulolytic bacteria. In the case of the functional rumen bacteria which fermented starch, glucose and xylose, no advantage was gained by substituting dithionite for cysteine in the medium. Moreover, the accurate dosage of dithionite solution into the medium immediately before inoculation involved a considerable amount of labour; hence it was decided to revert to the use of cysteine in the media for these groups of bacteria.

Choice of redox indicator. From the figures quoted above for the redox potential of Hungate type media, it would appear that the indicator which has been most widely used in media for rumen bacteria, namely resazurine, is not well suited for the purpose. The normal potential of this indicator at pH 7·0 is $+51$ mV., and it will show its oxidized colour only after most of the cysteine in the medium has been oxidized. This might explain the observations of Bryant (1959) that the growth of *Bacteroides succinogenes* in a chemically defined medium was erratic as long as he relied on resazurine as an indicator of a suitably low redox potential in the medium. Only when he introduced more stringent anaerobic precautions, including the use of the more sensitive indicator indigodisulphonate (E'_0 -125 mV. at pH 7·0), did he obtain consistent growth of this organism. The suitability of indigodisulphonate as a redox indicator in different cysteine-containing media for rumen bacteria was tested in the present work. The concentration recommended by Bryant (0·0005 %) produced a visible colour depth of the oxidized form even in the thin layer of agar in the roll bottles, and no toxic effect has thus far been detected on the organisms developing on the different media. However, when tubes of an old batch of medium were exposed to the air, only a faint colour was produced; this limited the usefulness of the indicator in the preparation of large batches of medium to be stored for routine work.

For counts on the modified cellulose medium containing 0·003 % sodium dithionite, the use of an even more sensitive redox indicator seemed appropriate. Inspection of tables on redox indicators suggested that benzylviologen (E'_0 -359 mV. at pH 7·0) should be ideally suited for the purpose. In fact, this indicator was used with good success by Smith & Hungate (1958) in media for their rumen methane bacteria. However, it was found that the concentration used by these authors, namely, 0·001 %, tended to produce low counts on media containing glucose. Furthermore, this concentration of the indicator did not produce a visible depth of colour in the thin film of cellulose medium in the roll bottles and when the concentration was increased to 0·005 %, growth of the cellulolytic cocci was almost completely inhibited. Resort was made to indigodisulphonate (indigocarmine) added to the medium together with the dithionite solution immediately before inoculation.

Choice of rumen fluid supplement

Initially, counts were made on media containing rumen fluid from sheep on the same diet as the animal whose rumen flora was under examination. This was done to ensure that the growth factor requirements of the functional bacteria predominating under those dietary conditions would be satisfied. However, the application of this principle proved to be very tedious in practice since a complete set of media had to be prepared for every diet studied. It was decided therefore to test the feasibility of standardizing the medium by using rumen fluid from sheep fed a diet of lucerne hay on which the animal could be kept indefinitely. For the metabolic groups of rumen bacteria which ferment starch, glucose and xylose, and also for the cellulose digesters from teff hay-fed sheep, there was little difference whether the rumen fluid was derived from an animal fed teff or lucerne hay. A difference was found in the case of the cellulolytic cocci from sheep on lucerne hay: these grew faster and more abundantly on media supplemented with rumen fluid from animals on lucerne hay. It was therefore considered advantageous to standardize on rumen fluid from this source. In order to avoid introduction of appreciable quantities of extraneous growth substrates in the medium, the rumen fluid sample was withdrawn from the animals before morning feeding.

Sample treatment

It is realized that the method of preparing the sample of rumen contents by straining it through cheesecloth eliminates the 'fixed' bacteria, that is, those attached to the larger food particles. This leads to low counts of organisms such as the cellulolytic bacteria, a proportion of which is known to be located in cavities of the partially digested fibres. At present there is no method for removing these organisms quantitatively from the food particles without danger of impairing their viability, and any method which achieves only partial liberation of the fixed bacteria may be expected to result in a variation of counts which is no true reflexion of the changes occurring in the rumen. On the other hand, there is good reason to believe that the proportion of fixed to free organisms will be reasonably constant at a standard time after feeding. Since the method presented was intended for comparative counts of certain functional groups of rumen bacteria under different dietary conditions, rather than for obtaining absolute values for each group, the present system of sample treatment was chosen as the best practical compromise.

Tests of the method

Reproducibility of counts on a single sample. This was determined by running five parallel dilution series on cellulose agar, using a well-mixed sample of strained rumen contents from a lucerne hay-fed sheep as inoculum. Cellulose agar was chosen because it has been found that of the carbohydrate-fermenting functional rumen bacteria the cellulose digesters were the most difficult to cultivate and could, therefore, be expected to show the greatest variation in numbers. The sample was distributed over five 1 oz. screw-capped bottles which were completely filled and immediately chilled in ice in order to prevent changes in the composition of the bacterial population in the time interval between the withdrawal and the processing of the sample. For the same reason the subsamples were processed by a team of four workers, the operations of dilution, mixing, distribution and gassing being repeated over and over by the same workers to minimize the effect of personal errors. In this way, the dilution series from the last subsample was ready for incubation within 75 min. after collection of the sample.

The colony counts after 14 days of incubation of the four roll bottles of the sixth and seventh dilutions of each subsample are shown in Table 2. In this experiment there were no colonies in the sixth dilution of the second subsample, although there was good growth in the corresponding fifth and seventh dilutions. This is a very rare occurrence and may have been caused by contamination of that particular bottle with a toxic substance. The decrease of numbers of colonies from the sixth to the seventh dilution is in good agreement with the expected tenfold decrease. Moreover, considering that the growth of the cellulolytic rumen bacteria *in vitro* is very much dependent on the provision of their special environmental requirements in the medium, the coefficient of variation of the results obtained by our method, namely, 3·95 %, compares very favourably with the 7·15 % reported by Cook & Steel (1959) for dilution series of an *Escherichia coli* suspension prepared by their second method, which corresponds to ours.

Table 2. *Replicate counts of cellulolytic bacteria in a sample of rumen contents from a sheep fed lucerne hay*

Subsamples: dilution $1/10^6$					Subsamples: dilution $1/10^7$				
1	2	3	4	5	1	2	3	4	5
20	0	21	23	16	2	1	3	3	3
23	0	27	21	16	0	3	3	0	1
20	0	15	20	19	1	3	2	1	3
23	0	19	19	26	2	4	2	1	1
21·5	0	20·5	20·75	19·25	1·25	2·75	2·5	1·25	2·0

Mean of 4 determinations at $1/10^6$ dilution	20·5
Standard deviation	0·81
Coefficient of variation between subsamples	3·95 %

Sampling error. In view of the fact that the rumen ingesta are not homogeneous (Pearson & Smith, 1943) and that it is difficult to withdraw repeat samples from exactly the same part of the rumen, it was considered necessary to determine also the magnitude of the variations in counts due to the sampling technique. A knowledge of the sampling error is of importance in the interpretation of the results of comparative counts run on different days. The plan of the experiment was similar to that of the previous one with the exception that five consecutive small samples were withdrawn from random positions within the rumen and were separately strained and collected in 1 oz. screw-capped bottles. The counts of cellulolytic colonies in the sixth dilution after 14 days of incubation are shown in Table 3. From these results it appears that the coefficient of variation between consecutive samples, 22·67 %, is considerably higher than that of replicate counts on the same sample. It is, however, of the same order as that found for differences in the chemical analyses of samples from different parts of the rumen (Pearson & Smith, 1943; Smith *et al.* 1956).

Variations with time of cellulose counts on a practical diet. The method outlined above was applied to the determination over a period of months of the number of cellulolytic bacteria/ml. rumen contents of a sheep receiving a daily ration of 1000 g. chaffed lucerne hay + 16 g. of a balanced mineral lick. On the sampling days, the drinking water was removed from the pen before morning feeding in order to decrease the effect of a variable dilution of the rumen contents with water. The samples were taken 2·5 hr. after feeding and processed immediately.

The mean values of the counts of cellulolytic colonies in the sixth dilutions are shown in Table 4, the figures being rounded off to the nearest whole number. The coefficient of variation between these results (75·2 %) is much higher than that found in the case of the five separate samples withdrawn at the same time. From Table 4 it appears that great fluctuations in counts of cellulolytic bacteria did occur within a short time interval. Work is in progress to test whether these fluctuations might have been caused by day-to-day variations in the proportions of fibre to protein in the chaffed lucerne hay.

Table 3. *Counts of cellulolytic colonies in five consecutive samples of rumen contents from a sheep fed lucerne hay*

Consecutive samples: dilution $1/10^6$				
1	2	3	4	5
18	15	18	27	17
20	9	16	—*	11
10	22	19	26	13
21	16	19	24	14
17·25	15·5	18	25·6	13·75

Mean of 5 determinations at $1/10^6$ dilution	18·03
Standard deviation	4·09
Coefficient of variation between consecutive samples	22·67 %

* Bottle broke.

Table 4. *Variation of counts of cellulolytic colonies on samples of rumen contents from a sheep fed lucerne hay taken over a period of time*

Date	Mean of colony counts at dilution $1/10^6$	Date	Mean of colony counts at dilution $1/10^6$
27. iv. 59	4	2. x. 59	3
29 iv. 59	6	10. xi. 59	15
1. v. 59	2	11. ii. 60	6
18. vi. 59	11	13. ii. 60	22
23. vii. 59	14	18. ii. 60	4
14. viii. 59	3		

Mean of 11 determinations	8·18
Standard deviation	6·15
Coefficient of variation	75·2 %

From the above it may be concluded that the method described in this paper is sufficiently sensitive to measure the fluctuations in the concentrations of the rumen bacteria which ferment the four main carbohydrate constituents of the diet under practical conditions. Compared with the dilution count technique of Wilson & Briggs (1955), the present method has the disadvantage of being more time-consuming and requiring much more stringent anaerobic precautions. On the other hand, it is much more precise and is capable of showing up smaller variations in the bacterial population of the rumen than is the dilution count technique. An additional advantage of our method is that it permits the study of colony shape and the microscopic examination of the colonies from the high dilutions of the sample. This has proved of value in the quantitative study of the change of predominant species within a functional group, following a change of diet. Furthermore, the thinness of the agar film in the roll bottles facilitates the isolation of the predominant species of the different functional groups. Applications of the method to studies on the composition of the bacterial population of the rumen of sheep on two practical diets and during a change from one diet to another will be published elsewhere.

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The Lysogenicity of Staphylococci isolated in Poland

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SUMMARY

Strains of staphylococci were tested for lysogenicity and different 'lysogenicity patterns' were defined on the basis of the phage lytic spectra. The test of lysogenicity was used to indicate the pathogenicity, and to complement the phage-typing pattern. None of the coagulase-negative strains (65) carried a phage. The lysogenicity patterns of 76% of the coagulase-positive strains (122 tested) were determined. With the method of phage typing 80% of the coagulase-positive strains of staphylococci were classified. By using both methods (phage-typing and the test of lysogenicity) the classification of 92% of coagulase-positive strains was possible. Differentiation of identical phage-typing patterns was obtained by using lysogenicity patterns.

INTRODUCTION

It has been reported that there is an association between the prophage state of staphylococci and (i) coagulase production (Karska, 1958); (ii) resistance to antibiotics (Wahl & Fouace, 1958); (iii) the phage-typing pattern (Smith, 1948; Lowbury & Hood, 1953; Rountree, 1956; Wahl & Fouace, 1958; Asheshov & Rippon, 1959; Rountree, 1959). Moreover, the transduction of staphylococcal bacteriophage calls attention to gene transfers accomplished by staphylococcal bacteriophage (Morse, 1959). These observations suggest that the introduction of lysogenicity patterns to the classification of staphylococci may facilitate the differentiation of these strains.

METHODS

The strains of staphylococci used came from various sources; 171 were isolated from children hospitalized with various diseases in the Research Institute of Mother and Child in Warsaw during 1955 to 1959, and 16 strains were isolated from members of the Institute staff. The pathogenicity of these strains were determined by the coagulase test. The coagulase-positive strains (122) were obtained from: swabs from throat or nose (60), swabs from ears (1), pus (31), urine (1), exudates from sinuses (7), puncture of pleura (13), bile (1), eye (1), blood culture (1), skin vesicles (1). The coagulase-negative strains (65) came from: swabs from throat or nose (55), swabs from ears (2), pus (4), urine (4), puncture of sinuses (1), eye (4), blood culture (1), swabs from vagina (2), tooth socket (2).

The twenty staphylococcal strains used to propagate the phages for routine phage typing were obtained from the National Collection of Type Cultures, Colindale, London, England. They are designated by the same number as the phages with the prefix PS. The phages obtained from Colindale were: 3A, 3B, 3C, 6, 7, 42E, 47, 52, 54, 55, 70, 73, 75, 77. Those obtained from the National Staphylococcal Phages Centre, PZH, Warsaw, were: 29, 52A, 53, 71, 79, 80*.

The strains of staphylococci were tested for: lysogenicity ('lysogenicity patterns'), coagulase production, phosphatase production, mannitol fermentation, pigment production, and phage-typing patterns according to Williams & Rippon (1952) and Rippon (1956).

The strains of staphylococci were tested for lysogenicity as follows.

The organisms were grown in nutrient broth (brain heart infusion broth; Narbutowicz, 1955) at 37° for 18 hr. Then 0·5 ml. of culture was flooded on the surface of an agar plate, dried and induced with ultraviolet (u.v.) radiation for 6 sec. at a distance of 42 cm. from a Hanovia lamp type 10204. The plates, protected from light, were put into a refrigerator for 24 hr. Subsequently 2 ml. broth were added, removed and centrifuged at 3000 rev./min. for 30 min. The supernatant fluids were tested by plating them on appropriate indicator strains. The results were read after 6–7 hr. at 37° and subsequently after 12 hr. at room temperature.

The strains that were not shown to be carrying a phage were tested by the cross-culture method (Fisk, 1942), induced by u.v. irradiation (Hewitt, 1952). As basal strains twenty propagating strains from Colindale and thirteen coagulase-positive indicator strains from our collection were used. The coagulase-negative strains were also tested with twenty-two coagulase-positive and 35 coagulase-negative indicator strains from our collection. The different sensitivity of basal strains to bactericidal activity of u.v. radiation made us use different times of irradiation. Thus the strains induced on basal strains PS 3A, 3B, 6, 7, 29, 42E, 73, and 80 were irradiated for 10 sec., while those induced on basal strains PS 3C, 42D, 47, 52, 55, 70, 71 and 77 were irradiated only 3 sec. Whenever strains PS 53, 54, 75 were used as basal strains, they were grown on an agar plate at 37° for 1 hr. Then the tested strains were plated on the basal strains, dried and induced by u.v. irradiation for 3 sec. In each case the distance from the lamp was 57 cm. The strains induced on basal strains isolated in our department were irradiated for 6 sec. at distance of 42 cm. from the lamp. After induction the agar plate was protected from light, and incubated at 37° for 24 hr.

When it had been ascertained that the cultures were lysogenic, a square of agar was cut out of the area of confluent phage lysis and put into a test-tube with 2 ml. broth. The culture was shaken and put into an incubator for 4 hr. and then centrifuged for 30 min. at 3000 rev./min. The supernatant fluid was plated on the basal strain as well as on the tested one.

The 'lysogenicity patterns' were designated by the lytic spectrum of liberated phages on the standard set of propagating strains. For example, when a lysogenic strain had liberated phages able to propagate on PS 80, PS 79, PS 73, the 'lysogenicity pattern' was designated as PS 80/79/73.

Bacteriophages may be classified by their plaque morphology, resistance to physical and chemical agents, susceptibility to specific antisera and cross-resistance tests, but the host range has proved the most useful for diagnostic purposes because of its technical simplicity. According to Rippon (1956) the propagating strains of the typing phages are suitable for the determination of the lytic group of newly isolated phages. However, it is evident that many different phages may lyse the same propagating strain.

RESULTS

No phage could be obtained from the 65 coagulase-negative strains. Induction was carried out three times on 20 propagating strains from Colindale and 70 strains from our collection. In contrast, 93 of the 122 coagulase-positive strains released phages. The analysis of the pathogenicity of these staphylococci gave the following results. Of the 65 coagulase-negative strains 2 produced phosphatase, 9 fermented mannitol, 9 produced golden pigment, 12 demonstrated phage-typing patterns. Of the 122 coagulase-positive strains 117 produced phosphatase, 121 fermented mannitol, 1 produced white pigment, 97 indicated phage-typing patterns. The results are summarized in Table 1.

Table 1. *Characteristics of* 187 *staphylococcal strains isolated in Poland in the years* 1955–59

Strains	65 coagulase negative strains	122 coagulase positive strains
Lysogenic	0	93
Produced phosphatase and fermented mannitol	1	116
Did not produce phosphatase but fermented mannitol	8	5
Produced phosphatase but did not ferment mannitol	1	1
White pigmented	56	1
Golden pigmented	9	121
Belonged to the phage group		
I	2	57
II	2	4
III	8	15
I/II	0	2
I/II/III	0	19
II/III	0	1
Not typable with phages	53	25

Lysogenicity and phage typing of staphylococci

In tests for phage-typing pattern on 109 strains the following results were obtained: 76 strains were typable with phages at their routine test dilution (RTD), and 33 strains were typable with phages in a concentration 1000 times stronger (1000 RTD). Coagulase-negative strains were for the most part resistant to the action of bacteriophages. Among the 65 investigated strains only 12 were able to synthesize these phages.

After investigation of 122 strains the phage pattern of 97 coagulase-positive strains was defined. The strains were able to synthesize from 1 to 8 various phages. The highest frequency of synthesizing activity was found with the following phages: phage 80 (54 strains), phage 54 (26 strains), phage 79 (22 strains). The phages 55, 29, 42E were synthesized only in exceptional cases.

The 'lysogenicity pattern' has been established in 93 coagulase-positive strains of the 122 tested. Phages liberated from 82 strains were able to propagate on indicator strains from Colindale and those released from 11 strains were able to propagate only on indicator strains from our collection.

Phages liberated from lysogenic strains lysed from 1 to 6 various propagating strains. Most frequently lysed were PS 80 (phages liberated from 38 strains), PS 7 (phages from 33 strains), PS 73 (phages from 31 strains). Strains PS 52A, PS 29 and PS 6 were lysed only exceptionally. Strains PS 29, PS 52, PS 3B, PS 55, PS 71 and PS 52E were never lysed by phages liberated from tested strains.

By means of the phage-typing method 80 % of coagulase-positive strains of staphylococci were classified, and 76 % by means of the 'lysogenicity patterns'. The percentage of classified strains increases when both methods are used. It was found that 14 of the 25 untypable strains liberated phages and 17 of the 29 not lysogenic coagulase-positive strains were typable by means of bacteriophages. By using both these methods 112 strains (92 %) were classified. Strains of uniform phage patterns can be divided into smaller units. Such differentiation of strains with a uniform phage pattern 80 into subgroups with various 'lysogenicity patterns' is illustrated in Table 2.

Table 2. *'Lysogenicity patterns' of* 38 *coagulase-positive strains of staphylococci with phage pattern* 80

Current number	'Lysogenicity pattern'	Number of strains
1	PS80	11
2	PS80/52A/7	1
3	PS80/79/73	1
4	PS80/3A/73	1
5	PS80/7	5
6	PS80/73	12
7	PS80/77	1
8	PS3A/73	1
9	Lysogenic on indicator strains of our collection	3
10	Not lysogenic strains	2

DISCUSSION

The introduction of the 'lysogenicity pattern' into the classification of staphylococci may be of use for the definition of pathogenicity of the strain, and as a supplementary analysis of the phage-typing method. In agreement with Karska (1958) there exists a relationship between the presence of the prophage in the bacterial cell and the coagulase production. This statement is based on the lack of ability, in our tests, to liberate bacteriophages in 100 % of 65 coagulase-negative strains, and the ability to liberate bacteriophages in 76 % of coagulase-positive strains (122 tested). Evidence was found that the larger the number of indicator strains the more probable was the determination of lysogenicity of coagulase-positive staphylococcal strains. The 'lysogenicity test' presents an additional criterion for the determination of the pathogenicity of staphylococcal strains and in doubtful cases this test may be used to supplement the methods applied hitherto. The parallel investigation of the 'lysogenicity pattern' and of the phage-typing pattern increase the number of classified strains and enable the division of uniform lytic groups into smaller subgroups. This division is an important factor for the investigations of epidemics and for the origin and course of outbreaks of infection due to staphylococci, because it allows the detection of differences among otherwise apparently identical strains.

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A New Type of Flagellar Variation associated with New Antigens in the Salmonella Group

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SUMMARY

A new type of phase variation which changes in one direction only has been noted in some members of the Salmonella group. This was found in association with three hitherto undescribed flagellar antigens, z_{43}, z_{45} and z_{46}, which are characterized. These occurred naturally with particular frequency in Salmonella group E and in cultures which possessed an alternate g, s, t phase. Two of these antigens, z_{43} and z_{45}, were found less frequently in other O groups and in organisms possessing other alternative phases. Although the antigens in question can be readily transformed to the alternate phases of the organisms in which they occur, these alternate phases could not be changed to z_{43}, z_{45} or z_{46}.

INTRODUCTION

The classification of the Salmonella group is based on the antigenic structure of the somatic and flagellar antigens of the serotypes of which it is composed. The structure is indicated by arabic numerals for somatic antigens, alphabetical letters for flagella in phase 1 and numerals or letters for flagella in phase 2. Salmonellas may fail to produce flagella; they may produce but one type of flagella, when they are called monophasic; or they may produce two types of flagella, being known as diphasic. Diphasic types may occur naturally, either in phase 1, phase 2, or a mixture of both. In the laboratory there are simple cultural techniques by which a diphasic organism in phase 1 may be changed to phase 2 and vice versa. One organism has flagella of one type only but, if it is diphasic, has the ability to produce progeny with flagella of a different type; the phase change occurs easily in either direction. The Salmonella group is divided into subgroups based on somatic O antigens. For example, subgroup E is composed of serotypes containing O antigen 3; many of the organisms which are the subject of this communication are classified in group E.

RESULTS

Recently, many investigations have been made into the occurrence of salmonellas in home-produced and foreign animal feeding-stuffs and fertilizers. Some of these materials have been imported from countries whose salmonella problem has received little attention, so there is no information as to the salmonella serotypes present in man and animals in the country from which they have come. In addition to new salmonella serotypes composed of known antigens, three undescribed flagellar antigens have been found to which the symbols z_{43}, z_{45} and z_{46} have been assigned. These three antigens were found in organisms of O group E, in association with flagella having the formula g, s, t. The particular interest of these new flagella was the ease with which the change from the natural z_{43}, z_{45} or z_{46} phase to the g, s, t phase occurred, but the complete inability, by using all known techniques, to reverse this change. The ability of each culture to revert from single colonies of the g, s, t phase to z_{43}, z_{45} or z_{46} forms was studied. In many of the cultures, multiple colonies were examined by serial transfer in g, s, t antiserum. In all, more than 150 such colonies were transferred periodically in this manner for 6 months. In no instance was reversion of the g, s, t antigens accomplished. It was evident that there was a very marked difference in the variation rate of the g, s, t phases and that of the z_{43}, z_{45}, and z_{46} phases.

The z_{43} flagellar antigen

Among motile cultures belonging to group E_4, O = 1, 3, 19, were a number which were agglutinated only in low dilution by *Salmonella wichita* phase 2 (z_{37}) antiserum and were unaffected by all other flagella (H) antisera then in use. An H antiserum prepared from one such culture agglutinated all similar cultures isolated in the United States and some of those isolated in England. The symbol z_{43} was assigned to the H antigens of these cultures. The z_{43} antiserum was absorbed with *S. senftenberg* to remove O agglutinins and added to semisolid medium for isolation of other phases which might be present in the organisms. When inoculated into this medium, all cultures which possessed z_{43} antigens migrated readily through it, and from the spreading growth organisms having H antigens g, s, t were isolated in each instance. In addition to 33 such cultures belonging to O group E_4, four others were recognized, all members of group E_1, O = 3, 10, and three cultures isolated from one lot of tankage (which is a protein by-product of abattoirs) had the T_1 (transitional) antigens described by Kauffmann (1956). The antigenic formulae of representative cultures were 1, 3, 19:g, s, t:z_{43}, 3, 10:g, s, t:z_{43} and T_1:g, s, t:z_{43}.

The z_{45} flagellar antigen

Among the E_4 group cultures isolated in England were seven which were not agglutinated by z_{43} serum. An H serum prepared from one of these agglutinated four of the cultures. The H antigens of these cultures were designated as z_{45}. One culture had the T_1 antigen. One culture of group E_1 contained z_{45} antigen. Finally, it was found that the stock culture of *Salmonella halmstad* (3, 15:g, s, t) contained a z_{45} phase. From all of the cultures which contained z_{45} antigens, g, s, t phases were easily isolated by passage of the cultures through semisolid medium which contained z_{45} serum. The antigenic formulae of representative cultures was 1, 3, 19:g, s, t:z_{45}, T_1:g, s, t:z_{45}, 3, 10:g, s, t:z_{45} and 3, 15:g, s, t:z_{45}.

The z_{46} flagellar antigen

One culture of group E_4 isolated in England contained neither z_{43} nor z_{45} antigen. An antiserum prepared from it agglutinated the homologous organism in high dilution but did not agglutinate z_{43} or z_{45} forms. To the antigen thus revealed the symbol z_{46} was assigned. This culture also yielded a g, s, t phase when passed through semisolid medium containing z_{46} antiserum. The formula was 1, 3, 19:g, s, t:z_{46}.

With the exception of three cultures isolated from one lot of infected poults which received feed contaminated with a salmonella strain of the same type, 1, 3, 19:g, s, t:z_{43}, all cultures were isolated from feeds, feed ingredients, or fertilizers. The sources of the cultures are summarized in Table 1.

Table 1. *Biochemical reactions and sources of cultures*

Antigenic formula	No. of cultures	H_2S	Tartrate			Citrate	Mucate	Source
			D	L	I			
1, 3, 19:g, s, t:z_{43}	2	+	+	−	$+^{8-10}$	+	+	Bone meal, degreased bones*
	23	+	+	×	$+^{2-5}$	+	+	Animal feeds and ingredients, poults**
	7	−	+	×	$+^{2-5}$	+	+	Feed ingredients**
	1	$+^{2}$	+	×	$+^{5}$	+	$+^{2}$	Feeding meat and bone*
T_1:g, s, t:z_{43}	3	+	+	×	$+^{2-5}$	+	+	Tankage (one sample)**
3, 10:g, s, t:z_{43}	3	+	+	×	$+^{2-5}$	+	+	Meat by-products**
	1	−	+	×	$+^{4}$	+	+	Meat by-products**
T_1:g, s, t:z_{45}	1	+	+	+	−	+	+	Bone chips (India)*
1, 3, 19:g, s, t:z_{45}	3	+	+	+	−	+	+	Bone meal, hoof and horn meal*
	1	+	+	−	−	+	+	Bone meal*
	2	+	+	$+^{5-7}$	−	+	+	River Humber (England)
3, 10:g, s, t:z_{45}	1	+	+	+	−	+	−	Fertilizer (Pakistan)*
3, 15:g, s, t:z_{45}	1	+	+	+	−	+	$+^{4}$	*S. halmstad*, stock culture
1, 3, 15:g, s, t:z_{45}	1	+	+	+	−	+	+	Bone meal*
1, 3, 19:g, s, t:z_{46}	1	−	+	−	−	+	$+^{3}$	Bone meal*
1, 3, 19:g, s, t	34	+	+	×	×	+	+	Divers sources**
	2	+	+	+	−	+	+	
	31	−	+	+	−	+	$+^{2-4}$	
	220	+	0	0	0	0	0	Divers sources*
	2	−	0	0	0	0	0	

0 Not tested.

+ Positive in 1 day. Superscript numbers indicate day of incubation on which positive reaction first observed.

× Late and irregular.

− negative.

* Isolated in England from products not imported from U.S.A.

** Isolated in U.S.A. or isolated in England from products imported from U.S.A.

Biochemical reactions

Both H_2S-positive and H_2S-negative cultures were included in the group and Dr F. Kauffmann (personal communication) suggested that it might be possible to separate them into *Salmonella newcastle* and *S. senftenberg* types. *S. newcastle* was an H_2S-negative type described by Warren & Scott (1930) which later was shown by Kauffmann & Mitsui (1930) to be identical with the H_2S-positive *S. senftenberg* type although Kauffmann (1941) found that they differed in their reactions on organic acids. Following this suggestion, the cultures were tested for H_2S production and action on organic acids. A number of monophasic cultures were included for comparison. The results are given in Table 1. The thirty-one H_2S-negative monophasic cultures listed in the table conformed to the biochemical pattern of *S. newcastle* while the thirty-four H_2S positive cultures which gave delayed positive reactions in L-tartrate were typical of the *S. senftenberg* type.

Both H_2S positive and H_2S negative types occurred among the 1, 3, 19:g, s, t:z_{43} forms, and the organic-acid reactions of the 1, 3, 19:g, s, t:z_{45} forms were not uniform; further, the 3, 10:g, s, t:z_{43} cultures were not identical in their reactions. Thus, it was not possible to correlate biochemical and serological results and divide the E_4 organisms into *Salmonella newcastle* and *S. senftenberg*.

Agglutination tests

The results of agglutination reactions of z_{43}, z_{45} and z_{46} antigens and their respective sera are given in Table 2. Phase 2 of *Salmonella wichita* (z_{37}) was included since it is slightly related to z_{43}. *S. senftenberg* (g, s, t), *S. simsbury* (z_{27}), and an 'induced' phase of *S. senftenberg* (z_{34}) also were included since all are connected in one way or another with the g, s, t complex (Edwards & Moran, 1947). It was evident that z_{43}, z_{45}, and z_{46} were distinct antigens which occurred naturally and which had little relationship one to another or to other known salmonella antigens. Absorption tests proved that the three z_{43} forms isolated in England from products not imported from the U.S.A. were identical. Likewise, all z_{43} forms isolated in the United States, or isolated in England from imported American products, were identical, even though some belonged to group E_1, some to group E_4, and some had T_1 antigens. The genetic significance of the various subgroups of group E is now in doubt since it is well known that lysogenicity plays a large part in their characterization. Experience indicates that T_1 antigens may be derived from either E_1 or E_4 cultures as well as from other O groups. The z_{43} antigens of the British and American strains, though closely related, were not identical. After cross-absorption a slight residue of agglutinins remained in the antiserum derived from the British strains and a considerable residue in the antiserum of the American strains.

A detailed analysis of the z_{45} antigens was not done. Antiserum was prepared from only one culture and when this was absorbed with the other z_{45} phases it was

Table 2. *Agglutination reactions*

		Sera						
Antigens	Source	g, s, t	z_{27}	z_{34}	z_{37}	z_{43}	z_{45}	z_{46}
g, s, t	*S. senftenberg* 3007*	25,600	<100	<100	<100	<100	<100	<100
z_{27}	*S. simsbury* 11077*	<100	3,200	<100	<100	<100	<100	400
z_{34}	Induced phase of *S. senftenberg* CDC.629*, obtained by passage through g, s, t serum	1,600	<100	6,400	100	<100	<100	100
z_{37}	*S. wichita* CDC.6093–52*	<100	<100	200	3,200	400	<100	<100
z_{43}	*S. senftenberg* Col. Sal. 2545/57*	<100	<100	100	800	12,800	<100	200
z_{45}	*S. senftenberg* Col. Sal. 655/58*	<100	<100	<100	<100	<100	12,800	<100
z_{46}	*S. senftenberg* Col. Sal. 2634/57*	<100	<100	<100	<100	<100	<100	6,400

* These numbers indicate the particular strain of salmonella used. The prefix indicates the laboratory in which the strain was first investigated—CDC meaning the Enteric Bacteriology Laboratories, Communicable Disease Center, U.S.A., Col. Sal. the Salmonella Reference Laboratory, Colindale, England. Strains with no prefix are those reported in the original descriptions of such serotypes.

evident that at least three distinct but closely related antigens were present in the z_{45} complex. These distinct antigens could not be correlated with the O antigens of the cultures.

DISCUSSION

It was stated that g, s, t phase could be isolated without difficulty from all organisms which occurred in nature in z_{43}, z_{45} or z_{46} phases. In some instances, both g, s, t and z_{43} phases were evident in cultures when they were received for typing. From such cultures colonies were isolated which were agglutinated either by g, s, t antiserum or z_{43} antiserum. Those which were agglutinated by z_{43} antiserum were readily transformed to g, s, t by passage through semisolid medium to which homologous antiserum was added. On the contrary, when the progeny of colonies which had g, s, t antigens were similarly treated they were immobilized and no change in H antigens occurred. In each instance in which a culture that was agglutinated by g, s, t antiserum was thought to have changed to z_{43} or z_{45} by passage through g, s, t antiserum, it was possible to plate the original culture and isolate z_{43} or z_{45} colonies. In other words, it was necessary for overt z_{43} or z_{45} antigens to be present in the cultures if they were to migrate through g, s, t antiserum.

The question naturally arose as to whether one was dealing with three different serotypes of group E_4, characterized respectively by antigens z_{43}, z_{45} and z_{46}, and whether these should be given distinct designations in the Kauffmann–White Schema. In this connexion, it should be emphasized that reversion of the g, s, t phases to z_{43}, z_{45} or z_{46} did not occur; if one is so unfortunate as to isolate a colony with g, s, t antigens it is not possible to determine whether the strain in question is truly monophasic, or whether an irreversible g, s, t form of a diphasic bacterium has been selected. Rapid fermentation of L-tartrate seems to be a characteristic of forms having z_{45} antigens, but even the small number of cultures examined was not uniform in this respect. Strains having z_{43} antigens utilized I-tartrate, though some were slow, whereas strains having z_{45} antigens failed to utilize it. Hydrogen sulphide production is of no value in their differentiation and, in fact, H_2S-positive and H_2S-negative forms were isolated from the same feed sample. It must be concluded that the biochemical reactions ordinarily used do not suffice to distinguish cultures which possess z_{43}, z_{45} or z_{46} antigens. One can but speculate as to how many of the supposedly monophasic cultures of *Salmonella senftenberg* were derived from cultures such as those described above. Considering these facts, the writers would classify all these cultures as *S. senftenberg*, but it must be kept in mind that cultures of *S. senftenberg* may fail to be agglutinated by g, s, t antiserum and may be completely in a z_{43}, z_{45}, or z_{46} form when isolated. The same facts apply to cultures of groups E_1 and E_2 which previously have been characterized as having only the H antigens g, s, t.

In addition to the cultures described here A. B. Moran & P. R. Edwards (private communication) noted z_{43} antigen in a culture of *Salmonella kingston* (4, 12, 27:g, s, t:z_{43}) and we found the same antigen in a culture of *S. worthington* having the formula 1, 13, 23, 37:z:1, w:z_{43}; also we encountered a culture of *S. havana* which had the formula 13, 23, 36:f, g:z_{45}. As in the cultures of group E, once the cultures were reisolated from single colonies of other phases, they could not be forced to revert to the z_{43} or z_{45} form. These observations indicate that z_{43} and z_{45} antigens eventually may be found rather widely distributed throughout the Salmonella group.

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The Properties of some Antigens of *Trypanosoma brucei*

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SUMMARY

A soluble trypanosomal antigen occurs in the serum of rats infected with *Trypanosoma brucei.* This 'exoantigen' is probably not a protein and is electrophoretically a slow-moving component in the serum. The exoantigen which is also present on the surface as an agglutinogen appears to protect the trypanosomes against adverse environment, preserving their infectivity for long periods *in vitro.* Rats and mice immunized with exoantigen are protected against infection with the homologous strain of *T. brucei.* Rabbits infected chronically with *T. brucei* form antibodies to exoantigen. On disintegration of the trypanosomes, in addition to exoantigen, 'bound' antigens are released. In the natural disease exoantigens induce antibodies which may be concerned with protection against further infection.

INTRODUCTIOM

Immunological investigations of the African trypanosomes have been largely confined to the application of serological testing techniques without the necessary understanding of the relevant immunological mechanisms. The complement-fixation test (Schochaers, Neujean & Evens, 1953; Rodhain, Valckeg & van Gidsenhoven, 1941; Pantizel, Lafaije & Duret, 1959; Fromentin, 1959), the cell adhesion phenomenon (Duke & Wallace, 1930; Wallace & Wormall, 1931), the agglutination test (Inoki, Kituana, Nakabayasi & Kurogochi, 1952; Soltys, 1957), the respiratory inhibition test (Desowitz, 1956, 1959*a*; Thurston,1958) and the precipitin reaction (Gray, 1960) are but some of the manifestations of a complex immunological system. The exploitation of these methods for the diagnosis of trypanosomiasis and for the classification of trypanosomes has led to confusing and contradictory results, probably owing to the multiplicity of antigenic systems involved. The analysis of the antigens of trypanosomes should elucidate some of these problems, and the present study is an attempt to investigate systematically certain aspects of the mechanisms of immunity in trypanosomiasis. A soluble trypanosomal antigen, called 'exoantigen' which plays a significant role in the immunology of trypanosomiasis has already been briefly described (Weitz, 1960). These investigations are described in detail below.

MATERIALS AND METHODS

Strain used. A monomorphic strain of *Trypanosoma brucei* (Shinyanga III) was used. It was isolated from cattle in Tanganyika in 1956 and maintained by rapid passage in mice until a method of keeping the living strain in frozen tubes (−70°) was described by Polge & Soltys (1957). The passage strain used for day-to-day experiments was renewed at intervals from a frozen stock. After one or two passages in mice no differences were detected between such strains and those which had been passaged in mice for prolonged periods. Blood from mice taken 2 days after infection caused a maximum parasitaemia and death on the 2nd or 3rd day in the mice into which it was injected.

Animals. White mice from a uniform stock and weighing about 12–17 g. were used for infection experiments. Antigens of trypanosomes were obtained from infected white rats weighing about 150–200 g. Antisera were prepared in 300–500 g. rats and in 2–3 kg. New Zealand Red rabbits.

Antigenic materials

Infected rat serum (IRS) was obtained from rats by section of the axillary artery under ether anaesthesia 3 days after infection with *Trypanosoma brucei*, the blood being pooled in 250 ml. centrifuge bottles. The serum was obtained after the blood had clotted at room temperature and the trypanosomes had been removed by filtration. The IRS was stored at −10° and then freeze-dried. Dilute IRS was also obtained when trypanosomes were washed and, after concentration, had properties identical to native infected rat serum.

Normal rat serum (NRS) was obtained from non-infected rats.

Trypanosome homogenates (TH) were made by bleeding 3-day infected rats into *c.* 5 vol. of Alsever's solution (Fig. 1). The erythrocytes were removed after centrifugation at 1800 rev./min. for 3 min. and the supernatant containing the trypanosomes was again centrifuged at 2000 rev./min. for 7 min. A 'medium' M.S.E. centrifuge (Measuring & Scientific Equipment, London) was used in all experiments. The supernatant fluid (Supernatant I) was removed and the sediment of trypanosomes was mixed with 10 vol. of Alsever's solution and centrifuged for 5–8 min. at 2000 rev./min. The resulting supernatant fluid (washing I) was removed and the process repeated (washing II). Finally, the sediment of trypanosomes was resuspended in about 1–2 ml. of saline solution and frozen in special tubes at −10° for disintegration in a Hughes (1951) press. The trypanosome homogenate was stored at +4° or freeze-dried. Supernatant I and washing I were pooled and concentrated by pressure dialysis to the original volume and, as noted above, used as IRS.

Antisera

Anti-IRS serum. Rats were injected intramuscularly in the hind legs with a series of 10 weekly doses of 0·5 ml. of IRS mixed in equal volumes with 10 % potash alum solution (pH 6·8) and bled by cardiac puncture 7 days after the last dose. The rats were given a further dose of IRS and bled 7–10 days later, the procedure then being repeated; although some rats died after heart puncture, the majority were bled 5 to 10 times at intervals of about 10 days. The sera from the bleedings of all the rats at the same stage of immunization were pooled and kept at −10° or freeze-dried.

Anti-trypanosome homogenate rat serum (Anti-TH rat serum) was similarly prepared, the rats being inoculated with 0·5 ml. of mixtures of 2 vol. of TH and 1 vol. of 10 % (w/v) potash alum solution.

Anti-trypanosome rabbit serum was obtained from rabbits infected by a single dose of living trypanosomes derived from infected rat blood. Twice washed trypanosomes were suspended in Alsever's solution in a concentration of 5×10^5/dose of 0·25 ml. Injections by the intravenous, subcutaneous or intracutaneous routes gave similar results. The serum was stored at $-10°$ or freeze-dried.

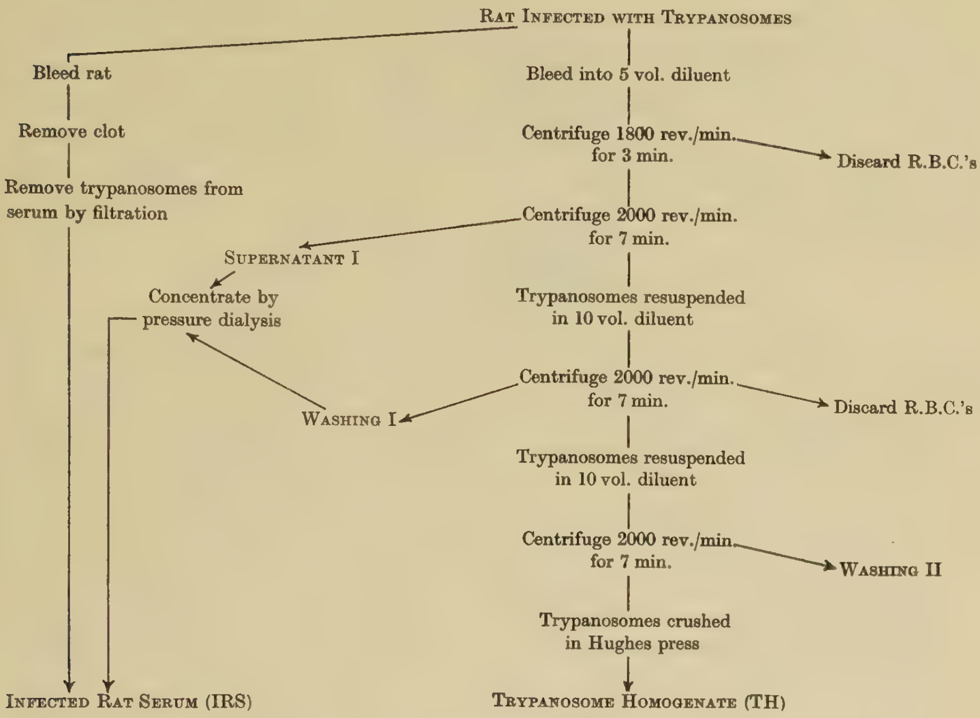
RAT INFECTED WITH TRYPANOSOMES
Bleed rat
Remove clot
Remove trypanosomes from serum by filtration
Bleed into 5 vol. diluent
Centrifuge 1800 rev./min. for 3 min.
Discard R.B.C.'s
Centrifuge 2000 rev./min. for 7 min.
SUPERNATANT I
Concentrate by pressure dialysis
Trypanosomes resuspended in 10 vol. diluent
Centrifuge 2000 rev./min. for 7 min.
WASHING I
Discard R.B.C.'s
Trypanosomes resuspended in 10 vol. diluent
Centrifuge 2000 rev./min. for 7 min.
WASHING II
Trypanosomes crushed in Hughes press
INFECTED RAT SERUM (IRS)
TRYPANOSOME HOMOGENATE (TH)

Fig. 1. Procedures in the preparation of infected rat serum and trypanosome homogenate.

Methods of testing

Mouse-infection experiments. Mice were usually infected with 0·25 ml. of suspensions of living trypanosomes by intraperitoneal injection; they were inspected twice daily and deaths recorded.

Agglutination tests. The agglutination test described by Soltys (1957) was done on slides examined microscopically under the $\frac{2}{3}$ in. objective, by mixing one drop of antiserum or control fluid with one drop of trypanosome suspensions derived from infected mice or rats bled into Alsever's solution containing 10 % horse serum.

Gel diffusion tests. Carried out at room temperature by the Ouchterlony method in 1·2 % agar in normal saline containing 1/10,000 thiomersalate.

Electrophoresis and immuno-electrophoresis. Electrophoretic analysis was made in starch gels using phosphate buffer (pH 7·7) according to Smithies (1955) in an electrophoresis tank as described by Kohn (1959). Up to six different samples were analysed in gels measuring 15 cm. × 10 cm. by inserting a piece of filter paper soaked in the sample and passing a current of 150 V at 35 mAmps for $5\frac{1}{2}$ hr. The starch gel was sliced in the horizontal plane, the inner surface of one portion being stained with naphthalene-black and decolorized in acetic acid + methanol + dist. water mixture (1 + 1 + 1).

For immuno-electrophoretic tests starch gels were divided into individual strips by cutting along both edges of each sample and slicing them horizontally. One portion was stained, while the matching slice was laid on a sheet of plate glass and 1·2 % molten agar was poured around the strip to the same thickness. When the agar had solidified, channels were cut out in the agar along the sides of the embedded starch strips and antiserum was poured in these channels. The plate was kept in a moist chamber to allow diffusion of the reagents. On completion of the reaction, the stained portion of the starch gel was laid over the embedded portion and the reacting zones were matched with the stained bands of the electrophorized samples. Alternatively, photographs of the stained strips were superimposed on photographs of the immuno-gel.

RESULTS

The presence of exoantigen in the serum of infected rats

Soluble trypanosome antigen ('exoantigen') in the serum of rats infected with *Trypanosoma brucei* was identified by precipitin tests with anti-IRS serum. IRS antisera, prepared in rabbits also precipitated with normal rat serum (NRS) but after absorption with NRS reacted only with IRS. To avoid using absorbed antisera, entailing difficulties due to excess of the absorbing antigen, rats were immunized with IRS, yielding antisera reacting with rat serum containing exoantigen but not with normal rat proteins. In agar gel anti-IRS rat serum gave a single line of precipitation with IRS and none with NRS. In constant antibody precipitin ring tests, the highest dilution at which IRS precipitated with anti-IRS rat serum indicated the amount of exoantigen in the samples. Various anti-IRS sera reacted at dilutions of 1/370 to 1/1280 of an average preparation of IRS. Comparisons of the titres of IRS obtained from rats at various times after infection suggest that the maximum amount of exoantigen was present in the serum soon before death.

The electrophoresis of NRS and IRS on starch gels stained with naphthalene black were qualitatively identical. Immuno-electrophoresis of these materials with anti-IRS rat serum showed that exoantigen in IRS was a slow moving component and just behind the serum γ-globulin component. No reaction occurred with the electrophorized NRS (Fig. 2). As exoantigen failed to stain with naphthalene black it is thought unlikely to be protein.

Some biological characters of exoantigen

It is the usual practice to infect animals with trypanosomes by the injection of infected whole blood, perhaps diluted in suitable fluid for convenience. However, when suspensions of such trypanosomes were washed before injection into mice, the infectivity was greatly reduced. The following experiments show that loss of infectivity was prevented by addition of exoantigen contained in IRS.

Living trypanosomes were obtained from infected mice bled into Alsever's solution. The erythrocytes were removed by centrifugation and the supernatant containing most of the organisms was centrifuged in conical tubes at 2000 rev./min. for 8 min. After removing the clear supernatant fluid, the sediment of trypanosomes was resuspended in 10 vol. of Alsever's solution and again centrifuged.

From the last sediment, suspensions of trypanosomes were made in Alsever's solution to contain 200, 20 and 2 million organisms per ml.. These were added in equal volumes to each of four dilutions of 1/2, 1/10, 1/20, and 1/40 of a five times concen-

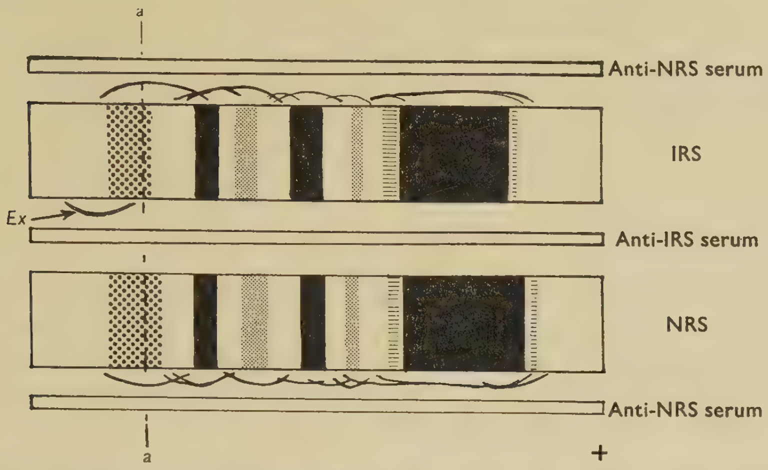

Fig. 2. Electrophoresis of infected rat serum and normal rat serum in starch gels and agar diffusion against antisera to normal and infected rat serum. *a*, a line of insertion of samples; *Ex*, line of reaction due to exoantigen.

Table 1. *Time to death of mice inoculated with graded doses of* Trypanosoma brucei

Inoculum			Death (days)										Mortality at 10 days	Average* time to death
T. brucei	Additive:	Concentration	1	2	3	4	5	6	7	8	9	10		
25×10^6	IRS	1/2	.	.	5	.	.	.	.	.	.	.	5/5	3·0
		1/10	.	5	.	.	.	.	.	.	.	.	5/5	2·0
		1/20	.	.	2	3	.	.	.	.	.	.	5/5	3·6
		1/40	.	.	1	4	.	.	.	.	.	.	5/5	3·8
	NRS	1/2	.	.	5	.	.	.	.	.	.	.	5/5	3·0
		1/10	.	.	3	.	.	.	.	.	.	.	3/3	3·0
		1/20	.	2	3	.	.	.	.	.	.	.	5/5	2·6
		1/40	.	2	3	.	.	.	.	.	.	.	5/5	2·6
25×10^5	IRS	1/2	.	.	3	2	.	.	.	.	.	.	5/5	3·4
		1/10	.	.	4	1	.	.	.	.	.	.	5/5	3·2
		1/20	.	.	.	3	1	1	.	.	.	.	5/5	4·6
		1/40	.	.	.	.	4	1	.	.	.	.	5/5	5·2
	NRS	1/2	.	.	.	.	.	4	1	.	.	.	5/5	6·2
		1/10	.	.	.	.	3	1	.	.	.	.	4/4	5·2
		1/20	.	.	.	.	3	2	.	.	.	.	5/5	5·4
		1/40	.	.	.	.	.	.	2	2	.	.	4/4	7·5
25×10^3	IRS	1/2	.	.	.	.	2	2	1	.	.	.	5/5	5·8
		1/10	.	.	.	.	1	4	.	.	.	.	5/5	5·8
		1/20	.	.	.	.	.	.	2	.	1	.	3/5	9·0
		1/40					No deaths						0/5	> 10
	NRS	1/2					No deaths						0/3	> 10
		1/10					No deaths						0/3	> 10
		1/20					No deaths						0/5	> 10
		1/40					No deaths						0/5	> 10

* In calculating the average time to death, survivors on the tenth day were given 11 days life.

trated IRS and NRS. The mixtures stood at room temperature for 1 hr. and 0·25 ml. of each mixture was inoculated into each of five normal mice. The numbers of trypanosomes injected into each mouse was thus 25 million, 2·5 million and 250,000 respectively in each group. The mice were observed for 10 days, deaths being recorded daily. The time to death (Table 1) depended on the number of organisms injected and at each dose level was directly proportional to the concentration of IRS or NRS in the inoculum. At all dose levels except the first which had the shortest possible incubation period, the mice which received IRS died earlier than those which received NRS. At the lower dose levels, although the mice which received trypanosomes with NRS survived at 10 days, at the higher concentration of IRS mice died in 5–9 days.

Nature of the increased infectivity of trypanosomes to which exoantigen is added

This increase in the infectivity of trypanosomes to which IRS was added might be due to agressin-like substances in IRS which enabled the trypanosomes to invade the host more readily. It is more likely that the apparent increase in infectivity was due to the more efficient preservation of trypanosomes *in vitro* as shown in the following experiment. Infecting organisms were prepared as in the previous experiment and the trypanosomes were added to equal volumes of undiluted IRS or NRS and plain Alsever's solution respectively. The three suspensions were maintained at room temperature for varying periods of 1, 2 and 5 hr. before inoculating a dose of 0·2 ml. of the mixture into each of 10 mice (see Table 2), so that each mouse received 750,000 organisms. Mice receiving trypanosomes with IRS or NRS incubated for 1 hr. died about the 2nd or 3rd day, but those receiving the same mixtures incubated for 2 hr. died about the 3rd or 4th day. When the mixtures were incubated for 5½ hr. before injection, although only one mouse died (8th day) of those

Table 2. *Time to death of mice inoculated with* 750,000 *living* Trypanosoma brucei *added to infected rat serum* (*IRS*), *normal rat serum* (*NRS*) *and Alsever's solution for different periods before inoculation*

Inoculum		Deaths (days)											
750,000 *T. brucei* added to equal volume of:	Time of incubation of mixtures before injection (hr.)	1	2	3	4	5	6	7	8	9	10	Mortality at 10 days	Average* time to death
IRS	1	.	.	7	2	1	.	.	.	.	.	10/10	3·4
NRS	1	.	.	8	2	.	.	.	.	.	.	10/10	3·2
Alsever's solution	1				No deaths							0/10	> 10
IRS	2	.	.	3	6	1	.	.	.	.	.	10/10	3·8
NRS	2	.	.	.	8	2	.	.	.	.	.	10/10	4·2
Alsever's solution	2				No deaths							0/10	> 10
IRS	5½	.	.	.	7	2	1	.	.	.	.	10/10	4·4
NRS	5½	.	.	.	.	.	.	.	1	.	.	1/10	10·7
Alsever's solution	5½				No deaths							0/10	> 10

* See Table 1.

which had received the trypanosomes with NRS, all the mice which had received 5½ hr. mixtures of trypanosomes with IRS died about the 3rd or 4th day. No mouse of any group died having received trypanosomes in Alsever's solution alone. Thus, in Alsever's solution, it appears that trypanosomes survive only for short periods while they survive for more prolonged periods in NRS and appreciably longer when suspended in IRS solution containing exoantigen, presumably the responsible component for the more efficient preservation of living trypanosomes *in vitro*.

Immunological characters of exoantigen

The experiments described above show the presence of exoantigen contained in IRS and its absence from NRS. The proof that the exoantigen is of trypanosomal origin and not merely an adventitious metabolite of rat serum resulting from infection lies in the following experiments.

The identification of exoantigen. Sera obtained from rabbits chronically infected with *Trypanosoma brucei* precipitated IRS in gel diffusion or the precipitin ring tests. The lines of precipitation with IRS are identical with the precipitation lines caused by the interaction of IRS and anti-IRS rat serum. No precipitate occurred with NRS. Moreover, rabbit sera absorbed with IRS no longer precipitated IRS. Both antisera, therefore, have identical antibodies. Considering that the rabbits were infected with only a few thousand well-washed trypanosomes it would have been impossible for any contaminating antigen of rat serum to have been injected in the rabbits with the infecting organisms in quantities sufficient to produce antibodies; and indeed, the rabbit sera contained no antibody to NRS.

Table 3. *Time to death of mice immunized with IRS and NRS and challenged with varying doses of* Trypanosoma brucei

Mice immunized with	Challenge dose	Death (days)										Mortality at 10 days	Average* time to death
		1	2	3	4	5	6	7	8	9	10		
Normal rat serum (NRS) (control group)	*a.* Fresh *T. brucei* infected whole blood	.	2	13	.	.	.	.	.	.	.	15/15	2·9
	b. T. brucei 5 × 10⁶ with IRS	.	.	1	4	.	.	.	.	.	.	5/5	3·8
	c. T. brucei 5 × 10⁶ with NRS	.	.	.	.	.	3	1	.	.	.	4/5	7·2
Infected rat serum (IRS) (immune group)	*a.* Fresh *T. brucei* infected whole blood	.	.	.	.	.	12	7	.	.	.	19/19	6·4
	b. T. brucei 5 × 10⁶ with IRS	.	.	.	.	.	.	2	1	.	.	3/5	8·8
	c. T. brucei 5 × 10⁶ with NRS	No deaths										0/5	> 10

* See Table 1.

The trypanosomal origin of exoantigen was further demonstrated by the fact that anti-IRS, anti-TH rat sera and anti-TRS rabbit sera agglutinated living suspensions of trypanosomes *in vitro*. The agglutination is inhibited when the antisera are absorbed with IRS but not with NRS. The antibody against exoantigen was therefore at least in part responsible for the agglutination of trypanosomes and the exoantigen is an agglutinogen occurring on the surface of the trypanosomes. The non-agglutinability of trypanosomes washed in Alsever's solution and presumably free from exoantigen confirms this conclusion.

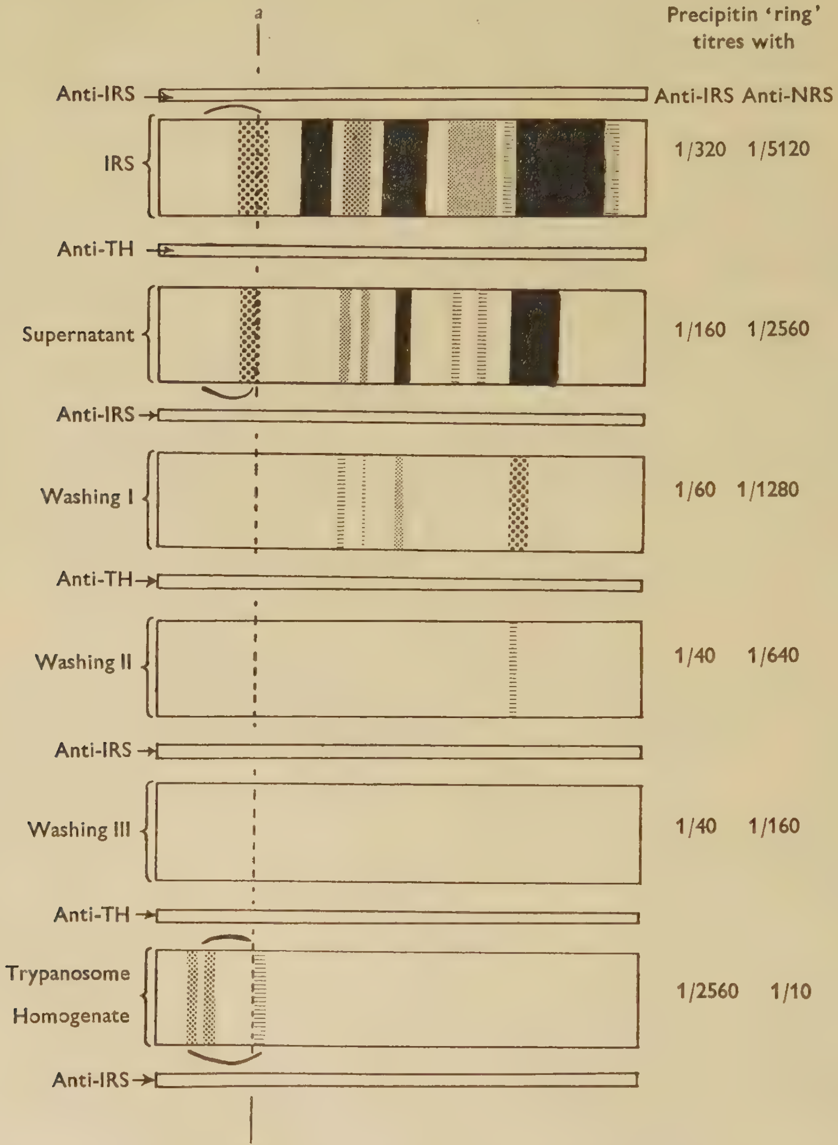
a
Precipitin 'ring' titres with
Anti-IRS
Anti-IRS Anti-NRS
IRS
1/320 1/5120
Anti-TH
Supernatant
1/160 1/2560
Anti-IRS
Washing I
1/60 1/1280
Anti-TH
Washing II
1/40 1/640
Anti-IRS
Washing III
1/40 1/160
Anti-TH
Trypanosome Homogenate
1/2560 1/10
Anti-IRS
a

Fig. 3. Starch gel electrophoresis of the materials in the preparation of trypanosome homogenates and their reactivity with anti-IRS and anti-THS sera. *a*, a line of insertion of samples.

Immunogenicity of exoantigen. Two groups of mice were given 4-weekly injections of 0·1 ml. intraperitoneally of five-times concentrated IRS and NRS respectively. A week after the last injection the mice of both groups were challenged with varying numbers of living trypanosomes to which IRS or NRS was added before inoculation. The time to death of the mice following challenge was longer in all groups of mice injected with IRS compared with those injected with NRS. Table 3 shows the time to death of the two groups of mice challenged with 5 million organisms with IRS and NRS respectively. The severity of the challenge was increased by the addition of IRS as compared with the addition of NRS and the protection of the groups immunized with IRS was related to the severity of the challenge. Thus, mice given the larger number of organisms with IRS added, died earlier than mice given less severe challenge when compared to the group of mice immunized with NRS. Mice immunized with NRS showed no protection at all as compared to normal mice. The mice in both groups which were challenged with 0·1 ml. of citrated fresh blood from trypanosome infected rats, being the most severe possible challenge, died very early, while the IRS immunized mice showed a significant protection against this challenge and on average died 3½ days later than the control group.

The specificity of exoantigen in relation to other trypanosomal antigens

No component staining with naphthalene black in starch electrophoresis was attributable to exoantigen, but the electrophoretic mobility of exoantigen was indicated by precipitation with anti-IRS rat serum in the corresponding immuno-gel (Fig. 2). In similar conditions disintegrated trypanosomes had two components situated on the negative side of the point of insertion and staining clearly with naphthalene-black (Fig. 3). The antigenicity of these components was established by means of rat antisera to trypanosome homogenates. The ability of trypanosome homogenates to precipitate with such antisera as well as with anti-IRS indicated they also contained exoantigen. However, in gel diffusion tests the lines of precipitation attributed to exoantigen were separate from those attributed to the two homogenate components indicating that exoantigen was distinct from the additional antigens derived from homogenates ('bound antigens'). Figure 3 shows the immunoelectrophoretic patterns of the solutions at various stages of preparation of the homogenates. Supernatant I, derived from the diluted infected blood after centrifugation, has the typical pattern of IRS and reacted with anti-IRS rat serum and anti-NRS serum, but not with anti-TH rat serum. Washing I had a similar but weaker pattern and reactions. Washing II showed no pattern and no lines of precipitation with either antiserum while TH showed only the two lines of the 'bound antigens' reacting with anti-IRS and anti-TH rat serum but not with anti-NRS serum. Thus, after the last washing and before the trypanosomes were disintegrated in the Hughes press, no more exoantigen could be washed off the protozoa, but on breaking them up considerable amounts of antigen were released, showing that relatively large amounts of exoantigens were still present in the cells.

DISCUSSION

The presence of soluble and bound antigens of the trypanosomes in infected rats has been established. Whatever the site of synthesis of the exoantigen in the trypanosomes, it is a soluble component occurring on the surface of the organism, whereas the bound antigens are not demonstrable unless the cells are disintegrated. The properties of exoantigen indicate the importance of this component in the process of infection and immunity.

Long ago, Marulli (1910) showed that when the serum of infected guinea pigs was injected at the same time as the infecting trypanosomes (*Trypanosoma brucei*) intraperitoneally, the infection in guinea pigs developed more rapidly than when the trypanosomes were injected alone. This effect may well have been due to the exoantigen from the infected serum. Exoantigen appears to be essential for the protection of the trypanosome against adverse environmental conditions *in vitro* and *in vivo*. Trypanosomes which have been thoroughly washed lose their viability and infectivity. Antibody to exoantigen is lethal to trypanosomes either *in vitro* or *in vivo*. This action may reduce them to a state similar to that of well-washed organisms by depriving them of the protective exoantigen. Moreover, the infection of rabbits with trypanosomes results in the formation of antibodies against exoantigen and to a less extent against the 'bound antigens'. The presence of such antibodies in the serum of cattle naturally infected with trypanosomes has already been demonstrated (Gray, 1960).

The agglutination of living trypanosome suspensions (Soltys, 1957) is dependent at least in part on the presence of exoantigen which is regarded as an agglutinogen, the agglutination test merely being a manifestation of one of the properties of the exoantigen. If the species specificity of the agglutination reaction is valid (Soltys, 1957), it is to be expected that the same degree of specificity would apply to the reaction of exoantigen with its antibody. Gray (1960) found antibodies in the serum of cattle naturally infected with *Trypanosoma vivax* only precipitating homogenates from several species of trypanosomes. The specificity of such cross-reactions in the field, as in laboratory infections, requires a careful study. For this purpose a more sensitive qualitative and quantitative determination of the exoantigen content of IRS based on the inhibition of agglutination of tanned erythrocytes, sensitized with IRS, has been developed. The method was not used in the experimental work described here and will be the subject of a further publication.

Thillet & Chandler (1957) found with *Trypanosoma lewisi* that suspensions of trypanosomes released soluble 'metabolites' in the suspending medium and that these were immunogenic and agglutinogenic. The preliminary experiments on exoantigen described here had been completed when Thillet & Chandler reported their findings. It is interesting that much of this work on *T. brucei* agrees with the observations of these authors on *T. lewisi*. To compare the bionomics of *T. lewisi* and *T. brucei* infections in laboratory animals may be confusing considering the function of ablastin in *T. lewisi* infections.

The investigations of the 'bound antigens' are clearly incomplete. Desowitz (1959*b*) showed that the homogenates of *Trypanosoma rhodesiense* had four electrophoretic components; the experiments described here indicate that the exoantigen component would not have been observed under the conditions of his experiments and the components he observed could, in fact, be attributed to contamination with rat serum. As none of the components he described was investigated immunologically, it is not possible to make comparisons between the components obtained from the strain of *T. rhodesiense* which he used and the strain of *T. brucei* used in these experiments. The strain and species specificity of the exoantigen and bound antigen require thorough investigation. It is not clear whether the various properties of exoantigen are different manifestations of a single antigenic component or are associated with a number of antigens. The present experimental knowledge suggests that exoantigen of the strain of *T. brucei* used is homogeneous.

Until the protection of mice immunized with exoantigen has been shown effective also against infection with trypanosomes by tsetse fly, the conclusions made here can only apply to infections by syringe inoculation in laboratory animals and the role of soluble antigen in field infection cannot be forecast at present.

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A Classification of the Klebsiella Group

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SUMMARY

Klebsiella strains are divided into six categories on the following reactions: fimbriation, production of gas from glucose at 37°, acid from lactose and dulcitol, methyl red test, acetoin production, citrate, urease, gluconate, malonate, lysine decarboxylase, growth in the presence of KCN and ability to produce gas from lactose at 44°. Five of the six categories are assigned specific rank, the sixth subspecific rank. The tests enable Friedländer's pneumobacillus to be distinguished from *Aerobacter aerogenes*. A new species, *Klebsiella edwardsii*, and a new subspecies, *K. edwardsii* var. *atlantae*, are named. Neotype strains of old species are proposed, holotypes of the new species and variety designated, and the catalogue numbers in the National Collection of Type Cultures and American Type Culture Collection given.

INTRODUCTION

Although the Klebsiella group is easily subdivided into serotypes by differences in the capsular antigens, a subdivision by biochemical tests into species is more difficult. The group comprises organisms that are non-motile, capsulate Gram-negative rods, and they have been named *Klebsiella pneumoniae*, *K. friedlaenderi*, *K. rhinoscleromatis*, *K. ozaenae* and *Bacterium lactis aerogenes* (synonym *Aerobacter aerogenes* Beijerinck). Two species, *K. rhinoscleromatis* and *K. ozaenae*, have characters that are reasonably distinctive, and the real problem is to decide whether it is possible to distinguish between *B. lactis aerogenes* and *K. pneumoniae* (*K. friedlaenderi*). This problem arises from the inadequacy of the early descriptions of these species for, as Edwards (1929) pointed out, Friedländer did not indicate whether his pneumobacillus fermented lactose or acted on milk.

Frankland, Stanley & Frew (1891) found that their strain (which they called Friedländer's pneumococcus) fermented glucose, sucrose, lactose, maltose, raffinose, dextrin and mannitol producing ethanol and acetic acid, but it did not ferment glycerol or dulcitol. Grimbert's (1895) strains fermented glycerol and dulcitol and produced lactic acid, and combining the findings of these workers Grimbert & Legros (1900) recognized two races distinguished by end-products of fermentation. However, they thought that separation into two species, *Bacterium lactis aerogenes* and Friedländer's bacillus, was unjustified, a conclusion supported by Edwards (1929).

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Duguid (1959) reopened discussion of the problem when he suggested that saprophytic *Klebsiella aerogenes* were fimbriate while pathogenic *K. pneumoniae*, *K. rhinoscleromatis* and *K. ozaenae* were non-fimbriate. This new approach seemed so hopeful that the morphological and certain biochemical reactions of 176 strains were investigated; the results are presented in this paper.

METHODS

Strains. In all, 182 strains were studied; 176 were regarded as klebsiellas as they had the following characters. Generally capsulate, Gram-negative, non-motile rods. Colonies moist and generally mucoid, not pigmented. Gelatin not liquefied. Catalase-positive; oxidase-negative. Glucose and many other sugars fermented, usually with the production of both acid and gas. Most strains fermented adonitol and inositol, and most were known to have capsular antigens of the Klebsiella group (Report, 1958). Included were:

(*a*) Strains received from Dr E. P. Snijders, Amsterdam, as serotypes A–F (Goslings & Snijders, 1936) later redesignated 1–6 by Kauffmann (1949).

(*b*) Type strains of serotypes 7–69 from Dr Ida Ørskov, Dr P. R. Edwards and Dr P. N. Edmunds.

(*c*) Many strains of serotypes 1–6, and 30 strains of respiratory origin (serologically unselected) from Dr P. R. Edwards.

(*d*) Strains deposited in the National Collection of Type Cultures with such names as *Bacillus lactis aerogenes*, *Bacterium aerogenes*, *B. capsulatus*, *B. pneumoniae*, *B. ozaenae* and *B. rhinoscleromatis*. Among this group of strains was NCTC 204 which in the 1st edition of the Catalogue (1922) was described as 'Probably original Friedländer strain'.

For comparison, 6 other strains with IMViC reactions − − + + were studied:

NCTC 9735, a motile strain with the capsular antigen of Klebsiella serotype 47.

NCTC 10,006, type strain of *Enterobacter aerogenes* Hormaeche & Edwards.

NCTC 10,005, type strain of *Enterobacter cloacae* (Jordan) Hormaeche & Edwards.

NCTC 9529, a motile, slow gelatin-liquefying strain from water; isolated at the Metropolitan Water Board Laboratory.

NCTC 9741, 9743, motile, rapid gelatin-liquefying strains, isolated by Dr E. Hormache from wounds.

Biochemical methods used were, in general, those recommended in Report (1958). The exceptions were as follows:

Indole: oxalic acid paper method of Holman & Gonzales (1923).

Methyl red (MR) and Voges-Proskauer (VP; acetoin): strains were grown in glucose phosphate peptone medium for 5 days at 30°. Acetoin detected by Barritt's (1936) method.

H_2S production: detected by lead acetate papers over broth containing cysteine (Clarke, 1953).

Citrate: Koser's (1923) medium was inoculated with a straight wire from a saline suspension. Positives were confirmed by subculture.

KCN: the method was that used in the Salmonella Reference Laboratory, Colindale (Dr Joan Taylor, personal communication), a modification of that described by Møller (1954).

Malonate and phenylalanine: using the combined medium of Shaw & Clarke (1955).

Decarboxylase activity: determined by Møller's (1955) and Falkow's (1958) methods but, as these sometimes differed, only the results of Møller's method are recorded here.

Other tests, not mentioned in Report (1958) were:

Fimbriae: (Duguid, Smith, Dempster & Edmunds, 1955; also called 'pili' by Brinton, 1959) observed by the electron microscope in gold-palladium shadowed films of washed, formaldehyde-fixed bacilli from broth cultures.

Adhesins: MS adhesin was demonstrated by haemagglutination tests with guinea-pig red cells with and without 0·5 % D-mannose, and MR adhesin by similar tests with tanned ox red cells (Duguid, 1959). These examinations were made on serial aerobic cultures grown in nutrient broth for 48 hr. at 37° and in a repeat series of cultures at 30°.

Capsules: demonstrated in wet India ink films of the bacilli grown in broth and on an excess-sugar agar medium at 37° (Duguid, 1951).

Gluconate oxidation: by the method of Shaw & Clarke (1955), using as control, medium without gluconate to show the absence of reducing sugar in the capsular material.

44° *test*: inoculated tubes of MacConkey broth were held in a water bath at 44(±0·1)° for 48 hr. Gas in Durham's tube was recorded as positive; acid without gas was recorded as negative (Report, 1935).

Fermentation v. oxidation of glucose: method of Hugh & Leifson (1953).

Serological type was that indicated by the donor, but, in addition, Dr P. R. Edwards kindly typed several previously untyped strains.

RESULTS

In addition to the group characters described under Methods the following were common to the 176 klebsiella strains: glucose was fermented (Hugh & Leifson, 1953) and acid was produced from xylose, arabinose, rhamnose, maltose, trehalose, mannitol, sorbitol, and salicin. H_2S was produced from a cysteine-containing medium. None digested inspissated serum. Arginine and ornithine decarboxylase activity was not found. Characters common to all strains are of no value in subdividing the group, and were not considered in sorting the results of tests.

All strains except ten were found by the India ink method to be capsulate. The division between the capsulate and the non-capsulate strains was not clear-cut. Thus, certain strains produced visible capsules on only a proportion of rods; a few were capsulate only on excess-sugar medium or produced very soft, indefinite capsules, doubtfully distinguishable from loose slime.

In sorting the characters of the 176 strains three distinct patterns were at once discernible; these corresponded to *Bacterium lactis aerogenes* (referred to as A), *Klebsiella rhinosleromatis* (R), and *K. ozaenae* (O).

Table 1. *Frequency of reactions of* 176 *klebsiella strains divided into six categories*

Approximate percentages in parentheses.

	A	Pa	Pb	Pc	R	O
No. of strains	129	7	9	5	6	20
Fimbriae	129 (100)	0	9 (100)	0	0	0
Gas from glucose	126 (97)	0	9 (100)	5 (100)	0	17 (85)
Lactose (acid) prompt	128 (99)	0	9 (100)	0	0	2 (10)
Lactose (acid) delayed	1	7 (100)	0	5 (100)	0	18 (90)
Methyl red (MR)	13 (10)	2 (28)	9 (100)	5 (100)	6 (100)	20 (100)
Voges-Proskauer (VP)	128 (99)	7 (100)	0	2 (40)	0	0
Citrate	129 (100)	5 (71)	9 (100)	5 (100)	0	14 (70)
Urease	126 (97)	7 (100)	9 (100)	5 (100)	0	8 (40)
Gluconate	129 (100)	7 (100)	4 (44)	2 (40)	0	0
Malonate	122 (94)	3 (43)	9 (100)	0	6 (100)	0
Lysine	127 (98)	7 (100)	9 (100)	5 (100)	0	3 (15)
44° test	71 (55)	0	0	0	0	0
Dulcitol (acid)	40 (31)	0	9 (100)	0	0	0
KCN	129 (100)	7 (100)	1 (11)	5 (100)	6 (100)	19 (95)

Strains which did not fit into these three categories were subdivided into a further three categories (Pa, Pb and Pc) on fimbriation, their ability to produce gas from glucose at 37°, on the speed of lactose fermentation, fermentation of dulcitol, methyl red test, acetoin production (VP), production of alkali in a malonate medium and growth in the presence of KCN. The use of a prototype Determinator (Cowan & Steel, 1960) was of help in sorting these strains into the different categories, and when this was done the 176 strains gave the reactions shown in Table 1.

Fimbriate strains often have adhesins and categories A and Pb could usually be distinguished from the other categories by tests for these characters. Of the 129 A strains, 120 had MR adhesin and 112 MS adhesin, whereas none of the 9 Pb strains had MR adhesin but all had MS adhesin. However, since the adhesins may vary independently and strains possessing both (as in A) may fail to show one of them in certain cultures (Duguid, 1959), they were excluded from further consideration as distinguishing tests.

A minority of the fimbriate strains were poorly fimbriate, their cultures showing not more than 5–25 % of bacilli with fimbriae; all 9 strains of category Pb were of this type. By analogy with other fimbriate bacteria (e.g. *Shigella flexneri* and *Salmonella typhi-murium*) we may expect that A or Pb might include a few non-fimbriate mutant strains, while Pa, Pc, R and O strains would in all cases be non-fimbriate (as *Shigella sonnei*). In fact, no strain with the biochemical reactions typical of A or Pb was found to be non-fimbriate, but we must accept the possibility of this.

To make a diagnostic table (Table 6) percentages shown in Table 1 were converted to symbols; below 25 % was recorded as −(negative), above 75 % as +(positive) and the intermediate percentages as *d*, to indicate that different strains gave different results. The word variable was avoided as *d* characters of the individual strains were generally stable.

Table 2. *Distribution of* 176 *strains among the six categories graded according to number of discrepancies*

	Category					
Discrepancies	A	Pa	Pb	Pc	R	O
None	106	7	8	5	6	12
1	18	0	1	0	0	7
2	3	0	0	0	0	1
3	2	0	0	0	0	0

The percentage values of *d* affect the worth of the diagnostic table. Since, by chance alone, many tests could be expected to give an equal number of + and − results, we can assume that a test of value (e.g. gas from glucose) will be either 100 or 0 % (or near), and that a test of no distinguishing value will be around 50 %. If we make our *d* broad (e.g. 25–75 %) our classification (and our distinguishing tests) will be firmly based, but we can expect many *d* characters, and only a few well differentiated categories. If we make the *d* range narrow, we should have fewer *d*'s and better differentiation between the categories, but unless we are prepared to accept a great many categories, a high proportion of strains would show several discrepancies.

Strains which did not fit exactly into one of the six categories we defined were

Table 3. *General characters of six klebsiella categories and the reactions of six closely related bacteria*

	A	Pa	Pb	Pc	R	O	9735	10,006	10,005	9529	9741	9743
Fimbriae	+	−	+	−	−	−	+	+	+	−	+	+
Gas from glucose	+	−	+	+	−	+	+	+	+	+	−	+
Lactose (acid)	+	(+)	+	(+)	−	(+)	+	+	+	+	−	−
Methyl red	−	*d*	+	+	+	+	−	−	−	−	−	−
V-P	+	+	−	*d*	−	−	+	+	+	+	+	+
Citrate	+	*d*	+	+	−	*d*	+	+	+	+	+	+
Urease	+	+	+	+	−	*d*	−	−	−	−	−	−
Gluconate	+	+	*d*	*d*	−	−	+	+	+	+	+	+
Malonate	+	*d*	+	−	+	−	+	+	+	+	−	−
Lysine	+	+	+	+	−	−	+	+	−	−	+	+
Arginine	−	−	−	−	−	−	−	−	+*	+*	−	−
Ornithine	−	−	−	−	−	−	+	+	+	−	+	+
44° test	*d*	−	−	−	−	−	−	−	−	−	−	−
Dulcitol (acid)	*d*	−	+	−	−	−	−	−	−	+	−	−
KCN	+	+	−	+	+	+	+	+	+	+	+	+
Gelatin	−	−	−	−	−	−	−	−	(+)	(+)	+	+
Motility	−	−	−	−	−	−	+	+	+	+	+	+
Differences from A	0	3	3	4	9	7	3	3	6	6	7	6

* The arginine dihydrolase system takes part in this reaction (Møller, 1955). (+) = delayed; *d* = some strains +, some −.

put into the nearest and the number of discrepancies recorded. When a character was described as *d* it could not contribute to the discrepancies shown by a strain. From this sorting the results shown in Table 2 were obtained. Eight indole-producing strains had all other characters typical of category A, and they were included in the 129 strains. From Table 2 it will be seen that the most of our 176 klebsiella strains fitted into the categories without showing more than one discrepancy; four had two, and two had three discrepancies.

Table 3 shows the reaction of the six closely related enterobacteria in the same distinguishing tests, and it also shows the reactions (arginine and ornithine decarboxylases, motility and gelatin liquefaction) which exclude them from the Klebsiella group. NCTC 10006, the type strain of *Enterobacter aerogenes* Hormaeche & Edwards, has the same characters as NCTC 9735, but the latter is of additional interest in possessing the capsular antigen of Klebsiella serotype 47. Both these strains differ from our A category in only three characters, and show a closer relation than the other four strains to the Klebsiella group.

Table 4. *Correlation of strain source with category*

	Source		
Category	Respiratory	Non-respiratory	Unknown
A	59	61	9
Pa	4	1	2
Pb	4	1	4
Pc	4	0	1
R	6	0	0
O	13	0	7
Totals	90	63	23

Table 5. *Correlation between fimbriation, serotype and category*

Category	Fimbriae	Serotypes
A	+	1, 2, 4–69
Pa	−	1, 2
Pb	+	3
Pc	−	1
R	−	3
O	−	4, 5, 6

Correlation of the sources of the strains with the six categories was made when this was possible, but the sources of some strains remained unknown even after Dr P. R. Edwards had searched his records for us. One of our main interests in this investigation was to try to pick out Friedländer's bacillus from the non-respiratory organisms that were culturally similar; we described sources, therefore, under these three simple headings: respiratory, non-respiratory and unknown. Table 4 shows the relations between source and our categories.

Duguid (1959) suggested a correlation between fimbriation and serotype and, since the serotype of most of the strains was known, it was possible to see that there was a correlation between our categories and these features (Table 5).

DISCUSSION

Most workers have maintained that it was not possible to distinguish between the saprophytic *Bacterium lactis aerogenes* Escherich (syn. *Aerobacter aerogenes* Beijerinck) and Friedländer's pneumobacillus, but Duguid's (1959) suggestion that this might be possible made us review the characters of these organisms in the light of recent advances in bacterial characterization. Thus by studying the morphological features revealed by electron microscopy and combining this with the results of certain biochemical tests we have been able to divide our klebsiella strains into six categories. Differences between the categories depend on combinations of different characters, not all of which will vary in the same way in the same strain. Thus, A and Pb have only three completely different characters (KCN, methyl red and VP) but the *d* character dulcitol in A is a + character in Pb; similarly gluconate is + in A and *d* in Pb and it is unlikely that a strain would show the less typical reaction in both characters.

Pa and Pc have only one absolute distinguishing character (the ability to produce gas from glucose at 37°) but *d* occurs in five positions in the Table 6 distinguishing characters. In practice it is not difficult to distinguish between these categories, and the five strains in Pc do not fit Pa at all well. Pc has several features in common with O but, again, in practice there is no difficulty in distinguishing the categories.

A point we have not stressed, because the information is not usually available, is that the different categories are mainly made up of different serotypes; thus while A has representatives of all known serotypes except type 3, our Pb strains are exclusively of serotype 3 with one non-capsulate (and therefore untypable) strain, our Pa strains all of types 1 or 2, and our Pc strains all of type 1. It is of course possible that had more strains of the higher serotypes been examined some might have been found to belong to biochemical categories other than A.

Strains of category O show more individual differences than those of the other categories, and four strains of serotype 4 failed to reduce nitrate. Our results, taken in conjunction with those obtained by Dubay (1959), who examined 150 strains of *Klebsiella ozaenae* of serotype 4, suggest that this category might be divisible into two subcategories. Serotype 4 strains are usually urease-negative, whilst O strains of serotypes 5 and 6 are often positive in this character.

This classification of the Klebsiella group is applicable whatever taxonomic rank the group may be assigned. In schemes where the group is given generic rank our categories will be species; if the group is regarded as a species, our categories will be subspecies. In the discussion of nomenclature which follows, and in the selection and designation of nomenclatural types, the Klebsiella group is regarded as a genus.

In collecting strains we excluded from our Klebsiella group all motile strains; these are better regarded as belonging to *Enterobacter* (Hormaeche & Edwards, 1960), *Serratia* or *Hafnia*. Neither did we include any gelatin-liquefying strains. We agree with Lautrop (1956) that the so-called *Klebsiella oxytocum* would be better removed from the Klebsiella group.

NCTC 204 is reputed to be one of Friedländer's strains; it is now non-capsulate and thus is not fully representative of the species *Klebsiella pneumoniae*. It belongs to category Pb, which can therefore be considered to represent that species. Whilst

NCTC 204 cannot be typed on the basis of its capsular antigen, Kauffmann (1949) found it to belong to O group 1; NCTC 5056 (also in our category Pb) belonged to this O group but other strains of capsule type 3 (in our category R) belonged to O group 2A. The designation of category Pb as *K. pneumoniae* is in line with the thinking of Henriksen (1952) who thought that dulcitol-fermentation was a character of the species. Since NCTC 204 is non-capsulate, we propose another strain CDC 298/53 (NCTC 9633, ATCC 13,883) as neotype of the species *Klebsiella pneumoniae* Trevisan.

With the type species characterized, we next turn to the identification of the other categories. A is made up of organisms that would be accepted the world over as *Aerobacter aerogenes* Beijerinck. An unexpected finding was that about 50 % of the strains produced gas from MacConkey broth at 44°. These 44°-positive IMViC – – + + strains are widely distributed, and have been named the 'yarn organism' (Mackenzie, Taylor & Gilbert, 1948); they correspond to Irregular type VI of Report (1935). Perhaps they are worthy of subspecific status, but this we would not propose without further study.

The binomial *Klebsiella aerogenes* was proposed and must be ascribed to a Subcommittee of the Society for Applied Bacteriology (Report, 1956). A type culture was not designed by the Subcommittee, and we have chosen and propose as neotype strain C37 isolated at the Metropolitan Water Board Laboratories from water (NCTC 8172, ATCC 13,882); this strain is 44°-negative.

Category O is *Klebsiella ozaenae* (Abel) Bergey *et al.* As this category is biochemically less homogeneous than the others, we do not at present feel justified in selecting a neotype culture. Further study of a larger number of strains may show that division into two varieties is desirable.

Category R is *Klebsiella rhinoscleromatis* Trevisan. We propose strain R 70 (NCTC 5046, ATCC 13,884) as the neotype culture.

We are left with the two categories Pa and Pc; these have many characters in common and we regard them as two varieties of one species. It is not possible to assign to them a specific epithet from the past, and Pa we name *Klebsiella edwardsii* after P. R. Edwards of Atlanta, who has done so much to establish the serological classification of klebsiellas. Since we are also making a variety of the species, the name becomes *K. edwardsii* var. *edwardsii* and we designate strain F1 (NCTC 5054, ATCC 13,886) as type strain. Pc becomes the second variety which we name *K. edwardsii* var. *atlantae* designating strain CDC 4610-53 (NCTC 9496, ATCC 13,887) as type strain.

Descriptions of the neotype and holotype strains are given in the Appendix.

The classification makes use of fimbriation, a character that can be detected directly only by those with access to an electron microscope. Alternatively, an almost certain indication of the presence or absence of fimbriae may be gained by testing 37° and 30° broth cultures for haemagglutination of guinea-pig red cells (MS adhesin) and tanned ox red cells (MR adhesin). However, the determination of this character is not essential for the identification of the species and subspecies defined, though all relevant information is helpful, and the more characters that are known, the clearer the identification becomes. Only those characters necessary for the differentiation of the species are included in the table for use in the Determinator (Table 6).

Any new scheme of classification should be compared with the old; our scheme supplements rather than replaces those based on serology (Julianelle, 1926; Kauffmann, 1949; Brooke, 1951; Edwards & Fife, 1952; Edmunds, 1954; Ørskov, 1954, 1955). It provides a means of characterizing species within the genus and, we hope, will help to bring classification of the Enterobacteriaceae into line with other groups of bacteria in which genera are split into species which in turn are subdivided into serotypes or other subspecific taxa.

Table 6. *Distinguishing characters of six categories*

Table for use in Determinator (Cowan & Steel, 1960).

	A	Pa	Pb	Pc	R	O
Fimbriae	+	−	+	−	−	−
Gas from glucose	+	−	+	+	−	+
Lactose (acid)	+	(+)	+	(+)	−	(+)
Methyl red	−	*d*	+	+	+	+
VP	+	+	−	*d*	−	−
Citrate	+	*d*	+	+	−	*d*
Urease	+	+	+	+	−	*d*
Gluconate	+	+	*d*	*d*	−	−
Malonate	+	*d*	+	−	+	−
Lysine decarboxylase	+	+	+	+	−	−
44° test	*d*	−	−	−	−	−
Dulcitol (acid)	*d*	−	+	−	−	−
KCN	+	+	−	+	+	+

A = *Klebsiella aerogenes* (Beijerinck) Subcom. Soc. Appl. Bact.; Pa = *K. edwardsii* var. *edwardsii* n.sp.; Pb = *K. pneumoniae* Trevisan; Pc = *K. edwardsii* var. *atlantae* subsp.n.; R = *K. rhinoscleromatis* Trevisan; O = *K. ozaenae* (Abel) Bergey *et al.*; (+) = delayed; *d* = some strains +, some −.

Table 7. *Characters of proposed neotype and designated holotype cultures*

	Klebsiella aerogenes	*K. pneumoniae*	*K. rhinoscleromatis*	*K. edwardsii* var. *edwardsii*	*K. edwardsii* var. *atlantae*
NCTC number	8,172	9,633	5,046	5,054	9,496
ATCC number	13,882	13,883	13,884	13,886	13,887
Serotype	64	3	3	1	1
Gas from glucose at 37°	+	+	−	−	+
Acid from lactose	+	+	−	(+)	(+)
Acid from starch	+	+	−	(+)	+
Acid from glycerol	+	+	(+)	+	(+)
Acid from dulcitol	−	+	−	−	−
Methyl red	−	+	+	+	+
VP	+	−	−	+	+
Gluconate	+	−	−	+	+
Malonate	+	+	+	+	−
Urease	+	+	−	+	+
Lysine decarboxylase	+	+	−	+	+
Growth in KCN medium	+	−	+	+	+
Growth in Koser's citrate	+	+	−	+	+
Simmons' citrate	+	+	−	+	+
Christensen's citrate	+	+	+	+	+
Fimbriae	+	+	−	−	−
MR adhesin	+	−	−	−	−
MS adhesin	+	+	−	−	−

Key to carbohydrate fermentation: + = positive in 1–2 days; (+) = positive in 3–14 days.

We wish to thank all those who sent us strains and especially Dr P. R. Edwards who serotyped for us many of our untyped strains.

APPENDIX

DESCRIPTIONS OF PROPOSED NEOTYPE AND DESIGNATED HOLOTYPE CULTURES

Common characters

Non-motile, non-pigmented, capsulate Gram-negative rods. Grow readily on ordinary media. Aerobic, facultatively anaerobic. Catalase-positive, oxidase-negative. Gelatin not liquefied. Indole not formed. Nitrate reduced to nitrite. Hydrogen sulphide produced from cysteine-containing medium. Phenylalanine deaminase and arginine and ornithine decarboxylases absent. Starch not hydrolysed. No gas from MacConkey broth at 44°. Acid from glucose fermentatively. Acid from arabinose, xylose, rhamnose, galactose, mannose, maltose, sucrose, trehalose, adonitol, mannitol, sorbitol, inositol and salicin.

Distinguishing characters are shown in Table 7.

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The Titration of Trachoma and Inclusion Blennorrhoea Viruses in Cell Cultures

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SUMMARY

A technique is described for titrating trachoma and inclusion blennorrhoea viruses by counting the inclusions formed in HeLa cell monolayers. The method compares favourably in accuracy with other techniques used for the assay of viruses and is more reliable than titration in the chick embryo yolk sac.

INTRODUCTION

Hitherto trachoma and inclusion blennorrhoea viruses have been titrated only by inoculating suitable dilutions of a virus suspension into the yolk sac of embryonated eggs and recording either the death or infection of the embryos. However, since chick embryos vary in susceptibility to these viruses, this method is not satisfactory. Sometimes, even after a large inoculum, infection of the egg cannot be demonstrated (Collier, Duke-Elder & Jones, 1958). The finding that some strains of trachoma and inclusion blennorrhoea viruses induce cytoplasmic inclusions in cell cultures suggested an alternative method of titration (Furness, Graham, Reeve & Collier, 1960). The present paper describes a technique for assaying these viruses by counting the cytoplasmic inclusions formed in monolayers of HeLa cells.

METHODS

Viruses. The TE 55 strain of trachoma virus (T'ang, Chang, Huang & Wang, 1957) and the LB1 strain of inclusion blennorrhoea virus (Jones, Collier & Smith, 1959) were used.

HeLa cells. Stock cultures were propagated in 250 ml. bottles containing 10 ml. medium. The medium consisted of Hanks's balanced saline solution with the addition of 5% (v/v) tryptic digest broth, 0·5% (w/v) lactalbumin hydrolysate, 10% (v/v) horse serum, 2% (v/v) rabbit serum and 200 μg. streptomycin/ml.; adjusted to pH 7·2–7·4 with 1·4% (w/v) $NaHCO_3$. Serum was heated at 56° for 30 min.

When monolayers of HeLa cells had formed, they were washed in phosphate-buffered saline (PBS) without calcium or magnesium and separated by 3 ml. 0·02% (w/v) ethylenediamine tetra-acetic acid in PBS (Lwoff, Dulbecco, Vogt & Lwoff, 1955). The cell suspension was diluted tenfold in medium and dispensed in 10 ml. amounts into bottles or 2 ml. amounts into Leighton tubes, each containing a coverslip (Bellco Glass Inc., Vineland, N.J., U.S.A.). The cultures were incubated at 37°.

Virus assay. When the HeLa cells had formed a monolayer in the Leighton tubes, the medium was changed and each of four tubes was inoculated with 0·1 or 0·2 ml. of a suitable virus dilution. After incubation for 72 hr. the coverslips were removed, washed in buffer pH 6·8, fixed in 95 % (v/v) methanol in water for 1 hr. and stained with Giemsa May-Grünwald (8 ml. Giemsa; 4 ml. May-Grünwald; 80 ml. buffer, pH 6·8). The cells were differentiated for 60–90 min. in buffer, dried at 37°, cleared for 2 min. in xylene and mounted in DePeX (G. T. Gurr and Co. Ltd.). The cells were examined at a magnification of × 240 and the inclusions counted. Only vesicles containing stainable material and distorting the nucleus were accepted as virus inclusions.

Estimation of inclusion forming units (IFU). By means of a micrometer slide, the area of a microscopic field was estimated to be 0·196 mm.^{2} at a magnification of × 240. The Leighton tubes had a rectangular well with a base measuring 11 × 39 mm., an area of 429 mm.^{2} This was partly covered by a coverslip measuring 9 × 38 mm. The monolayer formed a continuous sheet of cells over both the coverslip and the exposed surface of the well not covered by the coverslip. Thus, the area of the monolayer exposed to infection with virus was equivalent to the surface area of the well of the Leighton tube, i.e. 429 mm.^{2} The number of fields in this area equals

$$\frac{\text{total area of monolayer}}{\text{area of field}} = \frac{429}{0{\cdot}196} \quad \text{or } c.\ 2200.$$

Assuming random adsorption of the virus over the surface of the monolayer, the number of IFU/ml. is given by the formula

$$\frac{\text{no. IFU counted}}{\text{no. of fields examined}} \times 2200,$$

adjusted by a factor determined by the volume and dilution of the inoculum.

Disruption of HeLa cells by ultrasonic vibrations. To disrupt the cells and release virus, the infected cells were scraped into the supernatant medium and transferred to a 50 ml. centrifuge tube cooled in ice water. The suspension was treated for 4 min. with ultrasonic vibrations by inserting the probe of an M.S.E. Mullard ultrasonic disintegrator with a power output of 60 W. at a frequency of 20 kc/sec.

Partial purification of virus in yolk sacs. After homogenizing the infected yolk sac for 1 min. in an M.S.E. blender with 4–5 ml. sucrose + potassium glutamate solution (Bovarnick, Miller & Snyder, 1950), the suspension was clarified by centrifuging at 1500 rev./min. for 10 min. The supernatant fluid was then centrifuged at 10,000 *g* for 90 min. in an angle head at 4° to deposit the virus which was finally resuspended in 4–5 ml. sucrose + potassium glutamate solution.

RESULTS

Infection of cells. Monolayers of HeLa cells in bottles were inoculated with 1 ml. purified infected yolk-sac suspension. After incubation for 6–18 hr. the medium was changed and the cells re-incubated. Both viruses produced some inclusions within 3–5 days. After 6–7 days the cells were resuspended in fresh medium and used to inoculate further monolayers. After about three serial passages, almost all the cells contained inclusions; these heavily infected cultures were used in these experiments.

Preparation of virus suspensions. Preliminary observations indicated that not all the virus was released spontaneously from the inclusions. The use of ultrasonic vibrations (USV) and freezing and thawing as a means of releasing virus was therefore investigated. Microscopic observations showed that USV disrupted normal cells within 3 min. Infected cells were therefore treated for 4 min. to ensure complete destruction of the cells and release of virus. To study the effect of USV on the infectivity of the released virus, the suspension was titrated and again treated with USV for 4 min. There was no alteration in its infectivity (Table 1). There was a decrease, however, after more prolonged treatment. Three cycles of freezing in solid CO_2 + ethanol followed by rapid thawing in a 37° water bath caused a significant loss of infectivity (Table 1). Disruption by USV was therefore adopted as the method of choice.

Table 1. *Effect of ultrasonic vibrations (USV) and three cycles of freezing and thawing (F & T) on the infectivity of trachoma and inclusion blennorrhoea viruses in HeLa cell monolayers*

Virus	Treatment to release virus from cells	No. IFU/ml. suspension	Treatment of released virus	No. IFU/ml. suspension
Trachoma	USV	8×10^3	USV	$7{\cdot}5 \times 10^3$
	USV	3×10^5	USV	$1{\cdot}6 \times 10^5$
	USV	$9{\cdot}5 \times 10^4$	USV	$1{\cdot}1 \times 10^5$
Inclusion blennorrhoea	USV	$7{\cdot}4 \times 10^5$	USV	$6{\cdot}5 \times 10^5$
	USV	$5{\cdot}2 \times 10^5$	USV	$3{\cdot}7 \times 10^5$
Trachoma	F & T	$1{\cdot}2 \times 10^5$	F & T	5×10^4
	F & T	$4{\cdot}4 \times 10^4$	F & T	$8{\cdot}4 \times 10^3$

Appearance of inclusions. To ascertain that all inclusions counted at time of assay were derived from virus in the original inoculum, monolayers were infected with a suitable dilution of virus and incubated; at 24 hr. intervals, four coverslips were selected at random, stained and examined. Although some inclusions could be seen after 24 hr., they were not readily identified until after 48 hr. when they were about the size of the cell nucleus. By 72 hr. the inclusions had enlarged, but had not significantly increased in number, indicating that a second cycle of infection had not occurred and that all the inclusions were derived from virus in the original inoculum. As a routine, therefore, results were read after 68–72 hr. since the inclusions were most easily counted at that time.

Random distribution of inclusions. An area of thirty consecutive fields along the length of the coverslip was arbitrarily chosen as the 'standard area' in which inclusions were counted. To determine whether the number of inclusions found in any one 'standard area' was a representative sample of the whole monolayer, several coverslips were picked at random and the inclusions in four different standard areas counted (a total of 120 different fields/monolayer). The number of inclusions found in each of the four standard areas was remarkably similar (Table 2). They seldom differed from the average for the 120 fields by more than 10%. This suggested not only that an area of thirty fields was adequate for our purpose, but also that the inclusions were randomly distributed. Random distribution on any coverslip was confirmed by comparing the observed distribution of inclusions in each field with that expected from a Poisson series (Table 3) and testing the goodness of fit by the formula

$$\chi^2 = S\left\{\frac{(O-E)^2}{E}\right\},$$

where S implies summation, O observed number and E calculated number of inclusions/field. There was a reasonable fit.

Table 2. *Variations in the number of inclusions counted in different areas of the same monolayer of HeLa cells*

	Monolayer no.*				
	1	2	3	4	5
Four 'standard areas' of 30 consecutive fields	124	133	135	138	309
	114	131	127	150	383
	118	102	116	139	317
	134	129	116	136	367
Av. no./30 fields	122·5	123·75	123·5	140·75	344
Difference between av. no. in 30 fields and:					
highest no. in 30 fields	11·5 (9·4 %)	9·25 (7·5 %)	11·5 (9·3 %)	9·25 (6·6 %)	39 (11·2 %)
lowest no. in 30 fields	8·5 (7·0 %)	21·75 (17·6 %)	7·5 (6·1 %)	4·75 (3·4 %)	35 (10·2 %)

* Monolayers 1–4 inoculated with the same virus dilution.

Table 3. *Poisson series fitted to inclusion counts*

	Monolayer no.						
	1		2 and 3			4	
No. of inclusions/field	Obs.	Calc.	Obs.	Obs.	Calc.	Obs.	Calc.
0–1	6	10·2	8	4	9·7	6	6·2
2	17	18·2	14	17	16·8	16	12·1
3	31	22·9	23	26	22·0	15	19·1
4	20	23·4	31	31	23·3	18	22·3
5	23	19·2	17	20	19·2	23	21·0
6	12	12·6	16	11	13·2	17	16·4
7	4	7·5	6	7	7·8	15	11·0
8 and over	7	6·2	5	4	6·7	5*	6·5*
9 and over	—	—	—	—	—	5	4·9
No. fields counted	120	120	120	120	118·7	120	119·5
Av. no. inclusions/field	4·08		4·1	4·1		4·7	
Total no. inclusions counted	490 $\chi^2_{(6)}$ 7·75		495 $\chi^2_{(6)}$ 5·74	494 $\chi^2_{(6)}$ 9·11		563 $\chi^2_{(7)}$ 5·02	

* Eight inclusions only.

Optimum magnification for identifying inclusions. After incubation for 72 hr. inclusions can be recognized at magnifications of ×120 and ×240. The lower magnification gives a field 4 times larger than at ×240, and its use might be advantageous when there are few infected cells. Counts made at both magnifications were, therefore, compared and were consistently lower at a magnification of ×120 (Table 4). The higher magnification was therefore used as a routine.

Variations between replicates. The accuracy of titrations, using respectively 3 and 4 replicate coverslips, was compared. These results, together with the % standard deviation (% S.D.) and % standard error (% S.E.) are given in Table 5. The standard deviation was calculated from the formula

$$\text{S.D.} = \sqrt{\frac{S(x-\bar{x})^2}{N-1}},$$

where S implies summation, $x-\bar{x}$ is the difference of each replicate from the mean and N the number of replicates; the % standard error (% S.E.) was calculated as % S.D./$\sqrt{N}$. As would be expected, increasing the number of replicates did not greatly improve the accuracy of the method. The % S.E. was usually about ± 10 %, although it might be as high as ± 25 %; errors which are due to the inevitable sampling variations. When the S.E. is outside these limits it appears preferable to repeat the assay rather than attempt to increase accuracy by using more replicates.

Table 4. *Variations in the number of inclusions seen in the same monolayer at* × 120 *and* × 240 *magnifications*

	No. of inclusions in 30 fields at:		
	× 120 magnification		× 240 magnification. Inclusions counted in standard area
Monolayer no.	Inclusions counted	Counts corrected to standard area*	
6	34	8·5	11
7	45	11·25	19
8	92	23	30
9	140	35	47
10	142	35·5	51
11	309	77·25	103
12	317	79·25	120
13	367	91·75	126
14	383	95·75	141

* Thirty fields at × 120 equals four standard areas.

Table 5. *Effect of the number of replicates on the* % *standard error* (% S.E.) *and* % *standard deviation* (% S.D.)

Average of three replicates			Average of four replicates		
Av./30 fields	% S.D.	% S.E.	Av./30 fields	% S.D.	% S.E.
21	20	11·6	21	21·9	11
43	16·5	9·5	44	17·5	8·8
47	18·5	10·7	46	16·3	8·2
47	13	7·5	122	12·9	6·5
66	36·8	21·2	167	16·5	32·8
116	44·8	25·9	245	18·0	9·0
121	15·7	9·0	454	17·8	9·0
236	20·8	11·4			
395	8·3	4·8			
399	28·2	16·3			
417	42·9	24·8			
471	19·2	11·1			

These results indicated that within the limits tested (i.e. using four replicates, an average of 20–450 inclusions in 30 fields) the accuracy of the technique was not affected by the number of inclusions per field.

There was a linear relationship between concentration of virus and the inclusion count (Table 6).

Table 6. *Relationship between inclusion count and virus concentration*

Virus	Dilution factor	Count Av./30 fields	Count × dilution factor
Trachoma	10^0	430	430
	10^{-1}	61	610
	10^0	447	447
	10^{-1}	43	430
	10^0	395	395
	10^{-1}	44	440
	10^0	242	242
	$10^{-0.5}$	81	256
	10^{-1}	25	250
Inclusion blennorrhoea	$10^{-0.5}$	269	850
	10^{-1}	75	750
	$10^{-1.5}$	33	1040
	10^0	439	439
	10^{-1}	40	400

DISCUSSION

Before a virus can be studied in detail a quantitative technique for its assay must be developed. The feline and murine pneumonitis viruses have been titrated by counting the inclusions formed in explants of yolk sac (Weiss & Huang, 1954). Trachoma virus also induces lesions in such explants (Gordon, Quan & Trimmer, 1960). However, the use of HeLa cells is much less laborious. When a standard area of 30 fields is counted in each of four replicate monolayers at × 240 magnification, a standard error of approximately ± 10 % may be expected; this compares favourably with the results obtained by Weiss & Huang (1954). The slight increase in accuracy obtained by examining a greater proportion of the monolayer, or by using more replicates, does not offset the extra expenditure of time and materials.

Within the limits investigated, i.e. 20–450 inclusions in 30 fields, the same standard error may be expected. Thus each series of monolayers measures a relatively wide range of virus titre. However, only suspensions containing more than 7×10^3 IFU/ml. can be assayed by the standard technique. To titrate lower concentrations, the size of the inoculum must be increased or a greater area of the monolayer examined. This cannot be achieved by using an optical system with a lower magnification and a correspondingly greater field area, as there is a significant decrease in the number of inclusions identified at the lower magnification.

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Serological Typing of Strains of *Streptococcus faecium* and Unclassified Group D Streptococci Isolated from Canned Hams and Pig Intestines

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SUMMARY

A collection of 85 strains of *Streptococcus faecium* and unclassified group D streptococci isolated from canned hams and pig intestines was typed serologically. Seventy-seven of the isolates, which included two motile strains, were distributed amongst 15 types and 4 subtypes. The distribution of types was widespread, the same type being isolated from several different countries.

INTRODUCTION

Ingram & Hobbs (1954) and Ingram & Barnes (1955) showed that group D streptococci may cause serious spoilage in pasteurized canned hams, particularly as they are able to grow in hams stored at low temperatures. These organisms also occur sometimes in normal hams where their presence may indicate faecal pollution and a potential danger of food poisoning. Elimination of these occasionally surviving organisms by more extensive heat treatment of cans is not always practicable and must be done by determining and eradicating their source. This may be a direct infection from the animal gut or contamination during factory processing. Barnes & Ingram (1955) identified by physiological tests the faecal streptococci from canned hams as *Streptococcus faecium* and those predominating from pig intestines as *S. faecium* and unclassified group D strains, no true *S. faecalis* being found; whereas in bacon factories *S. faecalis* predominated (Barnes, Ingram & Ingram, 1956). Although this suggested that streptococci from canned hams might originate from guts of pigs, a more precise identification than that indicated by physiological characteristics was necessary; we have serologically typed some of the organisms from these sources. As spoilage in canned hams cannot be predicted it was not possible to compare the canned-ham isolates with those from the relevant pigs. The present work was intended to establish the serological types of strains from these sources, rather than to draw conclusions about their epidemiology. Such evidence will be reported later.

Skadhauge (1950) typed group D streptococci from human sources and demonstrated by agglutination tests the presence of heat stable (O) antigens and heat labile (K) antigens. He distinguished 16 different O antigens among the strains of *Streptococcus faecium* and 5 among the unclassified and *S. durans* strains. Sharpe & Shattock (1952) differentiated by agglutination, and confirmed by precipitin tests, 24 serological types among group D streptococci isolated from faeces of human infants. These included 4 types of *S. faecium* (types 14, 16, 18 and 24), 2 types of *S. durans*, and 7 unclassified types. A further type of *S. faecium* was described by Sharpe (1952), and another was isolated from the gut of the pig by Fewins, Newland & Briggs (1957).

METHODS

Eighty-five strains of group D streptococci previously isolated from canned hams and pig guts (Barnes & Ingram, 1955) and consisting of 56 *Streptococcus faecium* isolates and 29 'unclassified type I' isolates, were received from Dr E. M. Barnes (Low Temperature Research Station, Cambridge). Of these 71 had previously been characterized biochemically by Barnes & Ingram (1955) and the other 14 by Dr C. F. Niven (personal communication) and Lake, Deibel & Niven (1957). One isolate was from bacon, 28 were from 20 canned hams, and 56 were from 14 pigs (Table 1). The 'unclassified type I' strains (Barnes *et al.* 1956) resembled *S. faecium* except that they did not ferment mannitol or arabinose. The types described in the present work were at first defined by agglutination and precipitin typing; the 5 *S. faecium* types of Sharpe & Shattock (1952) and Sharpe (1952) were included for comparison.

Preparation of type sera. (*a*) *Formol treated vaccines.* Organisms grown for 18 hr. at 37° in 40 ml. 1·0 % (w/v) glucose Lemco broth (GLB) were washed twice in 0·85 % (w/v) saline and suspended in 10 ml. 0·25 % formalin (0·1 % formaldehyde) saline, and stored at 4°. Formalin-treated vaccines were used for preparing all sera unless specified otherwise. (*b*) *Disrupted organisms.* Saline suspensions of washed organisms grown in 10 ml. GLB medium for 18 hr. at room temperature were shaken on the Mickle tissue disintegrator (Mickle, 1948) for 1·5 hr. with carborundum powder (Shattock, 1949). A fresh preparation was made for each rabbit inoculation.

With formol-treated and disrupted organisms, rabbits were given a course of five intravenous inoculations of 1·0 ml. suspension at 3 to 4-day intervals. This usually resulted in a potent type serum. When it did not a further course of inoculations was given. Type sera were stored with thiomersalate (0·01 %, w/v) as a preservative. Small samples of sera for current use were stored at 4°; the main bulks of sera were kept in 2 ml. quantities at −20°.

Agglutination tests. Bacterial suspensions for agglutination were prepared from 4 to 6 hr. cultures incubated at 37° in GLB medium, the organisms washed and resuspended in the same volume of formol saline and stored at 4°. Such washed suspensions were more stable than formol-killed broth suspensions. Dreyer's agglutination technique was used, and results read after overnight incubation at 50°.

For agglutinin absorption the method described by Sharpe & Shattock (1952) was followed.

Precipitin tests. HCl extracts (Lancefield, 1933) were prepared as described by Sharpe & Shattock (1952). In the earlier part of the work cultures were incubated at 37°, but later incubation at room temperature was used (Elliott & Barnes, 1959) since in a few cases this resulted in extracts which reacted more strongly with the homologous type sera. Organisms from 10 ml. of GLB medium were enough to obtain a potent type extract. Tryptic digest extracts were prepared by Elliott & Barnes's (1959) method except that strains were grown in GLB medium instead of Todd-Hewitt broth.

Precipitin ring tests were used for typing, the tubes being read at intervals up to 10 min. A heavy precipitin ring was usually obtained in 3 min. or less with the homologous serum and extract.

Gel diffusion tests were made by Mansi's (1957) modification of Ouchterlony's technique.

For the absorption of precipitins four volumes of sera were absorbed with 1 vol. of packed streptococci previously washed and killed with formalin. Absorption was carried out at 37° for 2 hr., followed by overnight refrigeration.

Physiological tests. Recognized methods for the identification of group D streptococci were used. These included: ability to grow at 10° and 45°; carbohydrate fermentations; reaction in litmus milk; production of NH_3 from arginine; ability to split aesculin; growth on potassium tellurite agar; type of haemolysis (Shattock, 1949; Barnes, 1956; Barnes *et al.* 1956; Fewins *et al.* 1957). For detection of motility, 6 hr. cultures in GLB medium incubated at room temperature, after several transfers incubated at the same temperature, were examined by hanging drop.

Table 1. *Serological types and subtypes of strains of* Streptococcus faecium *and unclassified group D streptococci isolated from porcine sources*

Serological type	Type strain	No. of strains in type	Species of group D	Source of strains: Country of origin	Source of strains: Isolated from
16	N/H53	3	faecium	Holland, U.S.A., Canada	2 canned hams 1 bacon
26	CH1	6	faecium	Belgium	1 canned ham
	P12/6	11	Unclassified	G.B.	5 pigs
26A	CH3	4	faecium	Belgium	1 canned ham
26B	P15/6	3	Unclassified	G.B.	3 pigs
26C	P15/9	3	Unclassified	G.B.	2 pigs
27	CH12	1	Unclassified	S. Africa	Canned ham
28	HGH511	8	faecium	Europe, U.S.A., Germany G.B.	6 canned hams 2 pigs
29	P14/6	2	faecium	G.B.	2 pigs
30	N/GE4B	4	faecium	Holland, U.S.A., Poland	4 canned hams
31	N/H2	1	faecium	Holland	Canned ham
32	N/H4	3	faecium	U.S.A., Holland, Denmark	3 canned hams
33	P3	1	faecium	G.B.	Pig
34	P7	1	faecium	G.B.	Pig
35	N/R64	1	faecium	G.B.	Pig
36	P1/12	2	faecium	G.B., U.S.A.	2 pigs
37	P6/4	5	faecium	G.B.	3 pigs
37A	P4/16	13	faecium	G.B.	7 pigs
38	P20/5	2	Unclassified	G.B.	2 pigs
39	P16/5	3	Unclassified	G.B.	2 pigs
Not typed		1	faecium	Holland	Canned ham
		1	faecium	G.B.	Pig
		6	Unclassified	G.B.	5 pigs
Total		85			

RESULTS

Of the 85 strains of group D streptococci investigated, 77 were distributed amongst 15 types and 4 subtypes (Table 1). The types were numbered to extend the 24 types of Shattock & Sharpe (1952). Sharpe's (1952) serological type of *Streptococcus* *faecium* was numbered 25, and the 14 new types described here comprised types 26–39.

Agglutination typing. For the preliminary work agglutination tests were used. Thirteen types were designated, the results being confirmed by agglutination-absorption tests and by reciprocal absorption tests when necessary. Heterologous cross-reactions occurred but they were eliminated by absorption with appropriate absorbing suspensions. Sometimes sera had to be absorbed with several different strains to obtain complete specificity, which occasionally decreased the titres of the sera. The titres of unabsorbed sera were usually between 640 and 5120, although in a few instances higher or lower titres were obtained. With some of the types (16, 26, 28, 30, 32 and 37 A) antisera were prepared against several different strains within the type, to confirm that they were homologous. Suspensions of previously designated types of *Streptococcus faecium* (types 14, 16, 18, 24 and 25) were tested against these sera. Type 16 gave a specific reaction with one type antiserum prepared against a canned ham strain; this reaction was confirmed by absorption.

Table 2. *Relationships between serological types and subtypes as shown by precipitin reactions, using absorbed and unabsorbed sera*

Type 26 and subtypes 26 A, 26 B and 26 C

Reciprocal absorption tests

	Unabsorbed sera			Absorbed sera		
HCl extract	T 26	T 26 A	T 26 B	T 26 abs. T 26 A or T 26 B or T 26 C	T 26 A abs. T 26 or T 26 B or T 26 C	T 26 B abs. T 26 or T 26 A or T 26 C
Type 26	+	+	+	−	−	−
Type 26 A	+	+	+	−	+	−
Type 26 B	+	+	+	−	−	+
Type 26 C	+	+	+	−	−	−

Type 26 C gave similar reactions

Types 37 and 37 A

	Unabsorbed sera		Absorbed sera	
HCl extract	T 37	T 37 A	T 37 abs. T 37 A	T 37 A abs. T 37
Type 37	+	+	−	−
Type 37 A	+	+	−	+

Precipitin typing. Precipitin tests with the 13 type sera described above showed good agreement with the agglutination results. With HCl extracts and unabsorbed sera many non-specific cross-reactions were observed. However, it was possible to remove these and, with a few exceptions, to substantiate the same serological types as by agglutination, with confirmation by absorption or reciprocal absorption tests. The antiserum which reacted with type 16 antigen by agglutination test also reacted by precipitin test; reciprocal absorption tests with a serum prepared against the original type 16 strain confirmed that the canned ham type was identical with it. The main difference between the two methods of typing was that with type 26 and its subtypes only the subtype antigens (discussed later) were revealed by agglutination test, whilst the precipitin test also showed the presence of the common type antigen. With type 37 and its subtype, however, both methods showed the same antigenic pattern. Four strains (listed as untyped in Table 2) were typed by agglutination but could not be by precipitin typing. The specific agglutination reactions may have been due to thermolabile unstable K antigens (Skadhauge, 1950) present in agglutination suspensions but likely to be destroyed in the preparation of HCl extracts.

The precipitin method was used for the further work described here as it is more suitable than the tube-agglutination method for the large-scale typing survey contemplated for the future with porcine isolates. For such work, the precipitin test is easily and rapidly performed and the results are clear-cut. HCl extracts are very stable and can be kept for more than a year and still retain their potency, whilst unstable agglutination suspensions need constant rechecking and renewing. Also, comparisons could be made more readily with the results of other workers currently studying the serological typing of group D streptococci (Elliott & Barnes, 1959; Mr T. F. Medrek, personal communication) who are using precipitin typing.

Antisera were prepared against strains which had not reacted previously, and two more types were distinguished, making a total of 15 types. Some of the unabsorbed sera were completely specific, others required to be absorbed with organisms of cross-reacting strains. Sometimes absorption with organisms of more than one strain was necessary to obtain specificity. Absorption with a strain of *Streptococcus faecalis* did not remove all non-specific antibodies; thus group reactions seem unlikely to be responsible for the cross-reactions. Many of the unabsorbed sera gave such potent reactions that three different types could be pooled; this would be useful for the preliminary typing of large numbers of strains.

Subtypes. A subtype was regarded as being closely related to, but not identical with, the type strain, in having the same type antigen and also possessing an extra one. Extracts of each reacted with both sera, but on reciprocal absorption the antiserum against the main type had all the antibody removed, while that against the subtype retained its extra antibody (Table 3). Subtypes were differentiated in type 26 which had three subtypes, and in type 37 which had one.

Tryptic digest extracts. When tryptic digest extracts were used for ring precipitin tests with a number of types, positive reactions were obtained with specific sera. This method was only tried with 5 types, however, as the extracts obtained by this method were not as satisfactory as HCl extracts. The reactions with homologous serum was not so strong, and hazy cross-reactions, not previously observed, occurred with some heterologous sera. Elliott & Barnes (1959), however, used the capillary precipitin test and not the ring test; and the former may give more pronounced reactions with such extracts.

Distribution of serological types in canned hams and in guts of pigs

Table 1 shows the number of strains falling into each type and subtype. Only with type 26 did *Streptococcus faecium* and unclassified strains occur in the same type, reciprocal absorption tests confirming them as serologically identical. Only two types (26, 28) contained strains from both canned hams and pig guts. Of ten isolates from a single canned ham, six were type 26 and four were type 26A. Seventeen of the other 18 isolates, each from a different canned ham, were assigned to six further types. Among the 49 pig-gut strains typed were 10 different types. Nine of the 14 pigs sampled harboured more than one serological type of group D streptococci in the intestine. From one pig four different types (28, 33, 34, and untyped) were isolated.

Untyped strains. Eight strains remained untyped. Sera were prepared against six of these but with all of them many cross-reactions occurred in addition to the homologous reaction. Absorption with any of the cross-reacting strains or with a strain of *Streptococcus faecalis* removed the homologous as well as the heterologous antibody and rendered the sera inactive. Not all the HCl extracts of group D streptococci tested reacted with these sera, although they reacted with a group D serum, so that it was unlikely to be a group reaction.

Additional strains of Streptococcus faecium *examined.* As most of the canned ham isolates were of European origin, whilst the pig gut isolates were from British pigs, some strains of *Streptococcus faecium* from European pigs were examined to see whether they were more likely to be the same types as those found in the canned hams. Thirty-seven strains of *S. faecium* isolated and identified by Dr E. M. Barnes, from Dutch, Belgian and Danish pigs were typed serologically, by agglutination test only, against 11 of the 15 types. Only three strains fell into the types described here: two from Belgium into type 16 and another from Belgium into type 28. These results were confirmed by absorption. When type sera were prepared against three of the remaining untyped strains, seven further strains were typed.

Table 3. *Differentiating physiological characteristics of* Streptococcus faecium *and unclassified group D streptococci*

				Fermentation of					
S. faecium types	Motility	NH_3 from arginine	Rapid reduction of LM*	arabinose	sorbitol	mannitol	melibiose	sucrose	raffinose
16, 30, 31, 32	−	+	−	+	−	+	+	+	−
26, 26A, 28, 33, 34, 35, 37	−	+	+	+	−	+	+	+	−
29	+	−	+	+	−	+	+	+	± (sl.)
36	−	+	+ or −	+	+	+	+	+	−
37A	−	+	+	+	−	+	±	+	−
Not typed	−	+	+	+	−	+	±	+	−
Unclassified group D types									
26	−	+	+	−	−	−	+	±	−
26B, 26C	−	+	+	−	−	−	+	−	−
27	−	+	−	−	−	−	−	+	−
38	−	+	+	−	−	−	+	+	−
39	−	+	+	−	−	−	±	+	± (sl.)
Not typed	−	+	+ or −	−	−	−	±	+	±

± = some strains positive, others negative; sl. = if positive only slightly so.
* Rapid reduction of litmus milk = after 6 hr. at 37°.

Physiological characteristics of serological types

Biochemical and physiological tests confirmed the previous description (Barnes & Ingram, 1955) of these streptococci. Additional tests, for the ability to produce ammonia from arginine, presence or absence of motility and fermentation of a wider range of carbohydrates were also made. Table 3 shows the differentiating characteristics of *Streptococcus faecium* and unclassified strains; *S. faecium* is distinguished from the unclassified group by fermentation of arabinose and mannitol. The *S. faecium* serological types were all very similar: type 29 (two strains) was differentiated from the others by motility and inability to hydrolyse arginine; the two strains in type 36 fermented sorbitol, and some strains in type 37 A did not ferment melibiose. The unclassified types were even more homogeneous: some differences in the fermentation of sucrose and melibiose occurred, and an occasional strain (one of type 29 and two of type 39) fermented raffinose weakly. Type 26 included typical strains of *S. faecium* and also unclassified strains. Strains belonging to types 16, 27, 30, 31 and 32, and one of those in type 36 produced acid with slow or partial reduction of litmus milk (the usual reaction of *S. faecium*). Strains belonging to the other types, however, were exceptional in rapidly reducing the litmus milk (within 6 hr.); this is usually a characteristic of *S. faecalis*.

Precipitin reactions of the type sera with previous type strains

HCl extracts of type strains of the 25 serological types of group D streptococci of Sharpe & Shattock (1952) and of the 5 types described by Fewins *et al.* (1957) were tested against our 15 specific type sera. Apart from type 16, shown to be identical with one of our types, only three cross-reactions occurred: type 25 (*Streptococcus faecium*) extract reacted with type 27 serum; types 11 (*S. durans*) and 18 (*S. faecium*) reacted with type 34 serum. Agglutination tests done in the early part of the work had shown no reaction between suspensions of any of the previously designated *S. faecium* types and the new type antisera. Antisera prepared against types 11 and 18 gave no reaction with type 34 extract, and antiserum against type 25 gave no reaction with type 27 extract. Absorption tests, however, showed that relationships did exist (Table 4). With types 25 and 27, although type 27 extract did not react with type 25 antiserum, type 25 extract reacted strongly with type 27 antiserum. Organisms of type 25 were also capable of absorbing all the homologous antibody from the type 27 antiserum. A similar situation occurred with types 11, 18 and 34, but involving three strains (Table 4). It had been previously observed (Sharpe & Shattock, 1952) that precipitin tests denoted a one-sided relationship between types 11 and 18. Attempts to demonstrate a separate type antigen in extracts of type 27 and type 34 failed. With formamide extracts and tryptic digest

Table 4. *Serological relationships between different types*

Types 25 and 27

	Unabsorbed serum		Absorbed serum	
HCl extract	T25	T27	T25 abs. T27	T27 abs. T25
Type 25	+	+	+	−
Type 27	−	+	−	−

Types 11, 18 and 34

	Unabsorbed serum			Absorbed serum			
HCl extract	T11	T18	T34	T11 abs. T18 or T34	T18 abs. T11	T18 abs. T34	T34 abs. T11 or T18
Type 11	+	+	+	+	−	+	−
Type 18	−	+	+	−	−	+	−
Type 34	−	−	+	−	−	−	−

+ Denotes a strong reaction in 1 min.

extracts a similar result to that with HCl extracts was obtained. Ethanol precipitation (Shattock, 1949) only gave similar but stronger reactions, and no other type antigen was found in the ethanol-soluble fraction. Extraction of the acid-soluble fraction from organisms by gentle heating to 55° for 30 min. gave the same results as heating to 100° for 30 min. and did not reveal a heat labile antigen. With acetic acid extracts no reaction occurred. To try to stimulate the formation of antibodies which would react with extracts of type 27 and type 34, rabbits were inoculated for several courses of injections with vaccines of type 11, type 18 and type 25. A course of formol-killed organisms was followed by inoculations with organisms disrupted on the Mickle (1948) tissue disintegrator, to reveal any antigens 'deep' within the cell rather than on the cell surface. Such treatment did not, however, give rise to any additional reacting antibodies in the sera, other than weak non-specific reactions probably due to the formation of group antigen.

By quantitative absorptions, absorbing the sera several times with small quantities of organisms, it was possible with type 34 serum to obtain a weak reaction (+ in 15 min.) with the homologous organism only and not with extracts of types 11 and 18. With type 27 serum a relatively stronger reaction was obtained with the homologous extracts (type 27) than with the heterologous extract (type 25). An extract of another strain, 2766/1, previously reported as belonging to type 25 (Sharpe, 1952) did not react at all with type 27 serum, nor absorb the homologous antibodies from it.

The anomalous antigenic relationships described here were confirmed by gel diffusion tests. The one-sided reaction between types 25 and 27 may be due to a common antigen which is fully antigenic in type 27, but in type 25 is only a hapten, capable of absorbing antibodies and of reacting in the precipitin test but not of giving rise to antibodies. This is the type antigen in type 27 but probably just a minor antigen in type 25 strain S748, where it is present in its hapten state. In strain 2766/1 (also type 25) it is not present, and so cannot be the type antigen. A similar relationship may occur with the other one-way reacting types. Agglutination tests do not reveal these antigenic relationships.

DISCUSSION

Fifteen serological types were differentiated among 85 strains of *Streptococcus faecium* and other group D streptococci with similar physiological properties. This serological classification was based on the possession of type antigens characterized by serological specificity. Lancefield (1941) considered the type specific substance of groups D streptococci to be carbohydrate, and Elliott (1959, 1960) confirmed this, with the same strains of *S. faecalis* and *S. durans*. It seems likely, therefore, that the type antigen of *S. faecium* will also be carbohydrate; this requires confirmation.

Subtypes observed here were demonstrated previously in other group D streptococci by Sharpe & Shattock (1952). The early appearance in the antiserum of the subtype antibody and the fact that with agglutination tests the subtype antigen only may be involved in the reaction suggests that it is readily available on the surface of the cell.

Motile strains of group D streptococci, many from intestinal sources, have been reported previously (Ødegaard & Gardborg, 1953; Graudal, 1957*a*, *b*; Hugh, 1959) with characteristics similar to those of our type 29. They appeared to resemble *Streptococcus faecium* most closely, although some strains fermented raffinose. However only Graudal (1957*b*) has described a few strains unable, like our type 29, to hydrolyse arginine; these were all pigmented organisms. The antigen demonstrated by the precipitin test is presumably the heat resistant somatic antigen of Graudal (1957*a*). The thermolabile flagellar antigen would be destroyed by preparation of the HCl extracted antigen.

Variation of physiological characteristics within a serological type is to be expected and has been noted by other investigators (Sharpe & Shattock, 1952; Fewins *et al.* 1957). The latter authors found in one type a strain of *Streptococcus faecium* and also unclassified strains resembling the 'unclassified type 1' strains of Barnes & Ingram (1955), which were among the strains typed here. These unclassified strains of Barnes & Ingram are closely related to *S. faecium* except that they do not ferment arabinose and mannitol, and to α haemolytic strains of *S. durans*, except that they usually ferment sucrose and occasionally raffinose weakly (Shattock, 1955). The rapid reduction of litmus milk by some of the strains is, however, not typical of either of the above species. These organisms appear to be related to both *S. faecium* and *S. durans*, and the fact that some of them fall into the same serological type as strains of *S. faecium* emphasizes the similarity between the two species which is shown by physiological tests (Lake *et al.* 1957).

The different serological types are widely distributed, in some instances strains belonging to the same types (16, 28, 30 and 32) occurred in samples from several different countries. As the sources of the canned ham and pig-gut strains were not the same it could not be expected that the same types would occur in both sources. Only types 26 and 28 occurred in both canned hams and pig intestines, but as more strains of these types were tested than any other, except type 37, this is not considered to be significant. One of the additional strains examined from pigs fell into a type previously only found in canned hams (type 16). Although type 26 occurred in canned hams and in pigs the canned ham isolates were *Streptococcus faecium* and the pig isolates were unclassified. It is necessary, therefore, to correlate the serological type with physiological characteristics: the unclassified type 26 strains in the pig intestine could not be the source of *S. faecium* type 26 strains in the canned hams, unless the latter have gained the ability to ferment mannitol and arabinose, which appears unlikely.

It is evident that other serological types of *Streptococcus faecium* exist, as only ten of the 37 additional pig gut isolates could be typed with the type sera available. Sharpe & Shattock (1952) found that two of their types of group D streptococci from faeces of human infants were identical with two types previously described by Lancefield (1941). Elliott & Barnes (1959) also stated that these two types corresponded to two of Lancefield's, and that two further types of Sharpe & Shattock (1952) were identical with another Lancefield type and an isolate from bovine endocarditis. These workers isolated three of these types from chick caeca. We have found that a strain of *Streptococcus faecium* from Canadian Cheddar cheese, isolated and characterized by Mr P. S. Robertson, belonged to our type 26. Thus, some of the types of group D streptococci are prevalent in many different habitats. Collaboration between workers who are typing these organisms serologically is needed to prevent unnecessary replication of types.

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